Metabolic Engineering of Zymomonas moblis for Ethylene Production

Yan He
Biogas Institute of Ministry of Agriculture
Bo Wu
Biogas Institute of Ministry of Agriculture
Wei Xia
Chengdu University of Technology
Kun-Yang Zhao
Biogas Institute of Ministry of Agriculture
Qiong Tan
Biogas Institute of Ministry of Agriculture
Yao Qin
chengdu university
Pan-Ting Liu
Biogas Institute of Ministry of Agriculture
Qin-Hui Yu
chengdu university
Guo-Quan Hu
Biogas Institute of Ministry of Agriculture
Mingxiong He (hemxion@hotmail.com)
Biogas Institute of Ministry of Agriculture https://orcid.org/0000-0002-9780-6865

Research

Keywords: Ethylene, Ethylene-forming enzyme, Zymomonas mobliis, Corn straw, Cellulosic biomass

Posted Date: December 31st, 2019

DOI: https://doi.org/10.21203/rs.2.19779/v1

Abstract

Background: Biological ethylene production via the ethylene-forming enzyme (EFE) can offer a promising sustainable alternative approach for fossil-based ethylene production. The high stress tolerance of *Z. mobilis* make it as promising bio-ethylene producer.

Results: In this study, Heterologous expression of the *efe* gene in *Z. mobilis* successfully converted the non-ethylene producing strain into an ethylene producer. What's more, we systematically performed the effect of knocking out the competitive metabolic pathway of pyruvate and the addition of nutrients to the medium to improve the ethylene production in *Z. mobilis*. These optimization pathways and different substrate supplies resulted in higher ethylene productivity (from 1.36 to 12.83 nmol/OD_{600}/ml), which may guide future engineering work on ethylene production in other organisms to further improve ethylene productivity. Meanwhile, we obtained ethylene production of 5.8 nmol/OD_{600}/ml in strain ZM532-*efe* by using enzymatic hydrolysate of corn straw as the sole carbon source. This is also the first report on the production of ethylene from cellulosic biomass.

Conclusions: These results indicate that the engineered *Z. mobilis* show great potential for production of ethylene from cellulosic biomass in the future.

Background

Ethylene is the one of the world's largest fundamental chemical feedstock and widely used in petrochemical industry and consumer markets, such as synthetic rubber, fiber, spolyethylene and polyvinyl chloride for plastics [1]. There is an increasing awareness that the demand for these products continues to rise with the sharp increasing of the world's population [1]. China's ethylene yield has ranked second in the world. Since 2013, China's ethylene production has shown a steady growth trend. By 2018, China's ethylene production reached 18.41 million tons, up 1.1% year on year (China Petroleum and Chemical Industry Association). In the first quarter of 2019, the yield of ethylene is up to 5.06 million tons (China Petroleum and Chemical Industry Association). Currently, steam cracking with raw materials for naphtha and ethane are the most important ethylene production method [2]. However, the large amount of CO_2 and by-products produced during this process will not only aggravate global warming, but also cause serious pollution to water resources [2]. Therefore, to meet the ethylene demand, it is an irresistible trend of developing an environmentally friendly and sustainable ethylene production method via microbial biotechnology.As a plant endogenous hormone, ethylene can be produced biologically and used to modulate growth, metabolism and defense response to biotic or abiotic stresses. Ethylene is produced in varities of higher plants from methionine via 1-aminocylopropane-1-carboxylic acid (ACC) synthetase and ACC oxidase [3]. A number of microbes also can produce ethylene from 2-keto-4-methylthiobutyric acid (KMBA) using methionine as the substrate via a three-step reaction [4]. However, for the majority of plant-associated pathogens and symbionts, ethylene is spontaneously formed form α-ketoglutarate via the ethylene-forming enzyme (EFE) [5, 6], which is different from that of microbes and higher plants. The α-ketoglutarate –dependent (KGA) pathway has been widely used in a quantity of

hosts, such as *Escherichiacoli* [7-9], *Saccharomyces cerevisiae* [10], *Pseudomonas putida* [11], *Trichoderma viride* [12], *Trichoderma reesei* [13],, and *Cyanobacteria* [14-16]. Among all these feedstocks used for bio-ethylene production, renewable lignocellulosic biomass should be considered as candidate carbon sources. Actually, in the past two decades, these cellulosic biomass have attracted more and more attention for bioenergy utilization, especially for bioethanol production [17]. As only one *efe* gene can lead to produce ethylene from common metabolites, it is of great interest to study the heterologous expression of EFE in cellulolytic fungi and microbes. Chen *et al* reported the successful heterologous expression of an integrated *efe* gene from the *P. syringae* pv*. glycinea* driven by *pgk* promoter in *T. reesei*, rate of ethylene production is 0.716 μmol/gCDW/h when 2 % wheat straw were used as carbon sources [13]. Although the ethylene yield is lower than other *E. coli* and yeast hosts, which also provide a novel pathway for utilization of cellulosic biomass. However, there is currently an critical challenges in these hosts that they are notably sensitive to stress pressure, such as furfural and acetic acid derived from lignocellulosic hydrolysates [18-23], which will be a huge drawback in future with regard to industrial applications.

Zymomonas mobilis is a model strain for producing bioethanol and has became an increasingly important chasis, strain in the application of biotechnology via a unique ED metabolic pathway [24]. It shows some advantages of higher specific rate of sugar uptake, a broad pH range (3.5–7.5, especially low pH) [25] and so on, which made it an ideal platform for commercial-scale production of desirable bio-products, such as sorbitol [26], succinic acid [27] and butanediol [28]. In the previous studies, our lab and other groups have constructed many stress tolerant *Z. mobilis* strains, which can make it as promising bio-ethylene producer[29-32]. Therefore, biological ethylene production in *Z. mobilis* can offer a promising sustainable alternative approach for fossil-based ethylene production by introducing the ethylene-forming enzyme (EFE) from *P. syringae* pv. *phaseolicola* PK2 (the Kudzu strain), which catalyzes the conversion of the ubiquitous tricarboxylic acid cycle intermediate α-ketoglutarate into ethylene.

Recently, our group first converted the *Z.mobilis* into an ethylene producer via heterologous expression of *efe* gene in *Z. mobilis* and achieved stable ethylene production. We subsequently increased the ethylene production rate six-fold through modification of central carbon metabolism. We also attempted to measure the effects of the addition of nutrients to the medium on ethylene production. The addition of AKG will lead to higher ethylene production. Meanwhile, strain ZM532-*efe* obtained 5.8 nmol/OD_{600}/ml of ethylene production when we used enzymatic hydrolysis of corn straw as the sole carbon source, which provide a novel way for the utilization of cellulosic biomass. Herein, the engineered *Z. mobilis* show great potential for production of ethylene from cellulosic biomass in the future.

Results

Construction and identification of plasmid pEZ15Asp-*efe*

A 1,050 bp fragment of *efe* gene was obtained using P1,P2 primers. With P3, P4 primers and P5, P6 primers, respectively a 300 bp fragment of *pdc* strong promotor and terminator were further amplified. A

720 bp fragment of *gfp* gene was obtained using P7,P8 primers. P9, P10 primers were used to obtain a 3009 bp fragment of pEZ15Asp.

These fragments were connected by Gibson assembly, and then transformed into *E. coil* NEB β10 and screened on LB agar plates with 100 (μg/mL) spectinomycin. The recombinant plasmid pEZ15Asp-*efe* was double-digested by *Bgl*I and *EcoR*I to obtain two fragments (4161 bp and 1200 bp). As expected, the recombinant plasmid pEZ15Asp-*efe* used for EFE expression was successfully obtained. The structure of the plasmid pEZ15Asp-*efe* is shown in the **Fig. 1a.**

Construction of recombinant strain ZM4-*efe*

The plasmid pEZ15Asp-*efe* was electroporated into competent cell of *Z. mobilis*. Tranformants were screened on RM agar plates with 100 (μg/mL) spectinomycin. The result of the identification is that a 2409 bp sequence was obtained when recombinant strain ZM4-*efe* was used as template and P4,P7 were used as primers, while genomic DNA of *Z. mobilis* was used as template at the same condition without amplified fragments. These results indicated that the plasmid pEZ15Asp-*efe* was successfully transferred into *Z. mobilis*. What's more, green fluorescence was detected by Fluorescent Inverted microscope (**Fig. 1b**). The result indicated that the plasmid pEZ15Asp-*efe* was successfully expressed in *Z. mobilis*. Based on these results, the recombinant strain ZM4-*efe* was obtained.

Ethylene production of strain ZM4-*efe*

Experiments were conducted to test whether the strain ZM4-*efe* was capable of producing ethylene by cultivating in serum bottles with rubber stoppers and aluminum cap. The initial production of ethylene observed for strain ZM4-*efe* was 1.36 nmol/OD_{600}/ml when 30 ml cultures with a start OD_{600}=0.102 cultured for 18 h at 30℃ in serum bottles. While the wild-type strain ZM4 couldn't produce ethylene under the same conditions. Subsequently, we attempted to reduce the culture volume to 20 ml and 10 ml, and the ethylene production for two different volumes cultured by the same condition was respectively 3.03 nmol/OD_{600}/ml and 4.31 nmol/OD_{600}/ml, which did not decrease as expected but increased than previously working volume. What's more, the glucose concentration in the medium increased from 2 % to 5 %, and the ethylene production did not increase. This may suggest that there are other factors affecting ethylene production.

To investigate the causal links between cell density and ethylene production, a series of experiments were further conducted. More than three parallel experiments were set in each group. As shown in the **Fig. 2**, the initial OD_{600} was 0.1, and with the extension of the culture time, the OD_{600} value gradually increased. When cultured for 9 h, the OD_{600} reached the bottleneck period. The final OD_{600} value was 1.56. while the ethylene production did not change regularly. when OD_{600} was between 0.3-0.5, ethylene production reached its peak of 6.7 nmol/OD600/ml. And then with the prolongation of the culture time, there was no significant fluctuation in ethylene production.

Optimization of ethylene metabolic pathway

Based on our previous work, four single knockout strains of ZM1360 (Δ*pdc*), ZM1570 (Δ*pfl*), ZM1596 (Δ*adhB*), and ZM1237 (Δ*ldhA*) were already constructed [10]. The recombinant plasmid pEZ15Asp-*efe* was respectively electrotransformed into four single knockout strains, and four recombinant strains of ZM1360-*efe*, ZM1237-*efe*, ZM1570-*efe*, and ZM1596-*efe* were obtained. We tested the expression of GFP protein to reflect the expression of the target protein EFE. As shown in **Fig. 3**, there was no expression of GFP protein in wild-type ZM4, and there are two bands in all the five ethylene-producing strains. One band (28.8 kDa) was the size of the GFP protein, and the other band (70.8 kDa) was the size of protein of the fusion gene. Those indicated that *efe* gene was successfully expressed in all five strains.

A series of experiments were further conducted to test the ethylene production of strain ZM4-*efe*, ZM1360-*efe*, ZM1237-*efe*, ZM1570-*efe*, and ZM1596-*efe* cultured in RM (2% glucose). As expected, the ethylene production of four strains all improved production compared with strain ZM4-*efe*. The strains ZM1360-*efe* and ZM1596-*efe* significantly increased by 51.6 % and 41.3 %, were 8.3 nmol/OD_{600}/ml and 7.73 nmol/OD_{600}/ml, respectively (**Fig. 4c**). Similarly, ZM1570-*efe* and ZM1237-*efe* also showed higher ethylene production compared with the control strain ZM4-*efe*, were 8.13 nmol/OD_{600}/ml and 7.18 nmol/OD_{600}/ml respectively (**Fig. 4c**).

The expression of *efe* at the transcriptional level was explored when OD_{600} value of five ethylene-producing strains was about 0.4. As shown in **Fig. 5.** With the ZM4-*efe* as the control strain，the value of expression of *efe* for ZM1360-*efe*, ZM1237-*efe*, ZM1596-*efe*, and ZM1570-*efe* were 2.65, 2.07, 2.14 and1.668. The *efe* expression of the four single-gene knockout strains was higher than that of ZM4-*efe*. Combined with the ethylene production of four strains in **Fig. 4c**, we found that the strain ZM1360-*efe* with the highest *efe* expression had the highest ethylene production. However, the trend of expression of *efe* gene for other single-gene knockout strains was different from that of ethylene production.

Experiments of cell growth, glucose consumption and ethanol production for six strains were also perfomed. As shown in **Fig. 4 a, b and d**. When cultured for 9 h, the vaule of OD_{600} for wild-type strains ZM4 is 1.46, and for five recombinant strains was around 1.25. Among recombinant strains, the cell growth of ZM1360-*efe* is slightly superior to other recombinant strains. At this time, glucose consumption of the control group ZM4 was almost consumed, and the ethanol production reached 92% of the theoretical output value. After culturing to 12 hours, the vaule of OD_{600} for five recombinant strains is about 1.47, and the glucose consumption of five recombinant strain was exhausted, and the average ethanol production reached 92% of the theoretical output value. When cultured for 9 h 14 hours, the wild-type strain ZM4 and the five recombinant strains basically reached the same in terms of cell growth, glucose consumption and ethanol production, without significant differences.

Effect of additional additives on ethylene production

In addition to supplementing arginine and AKG, which was essential substrates for ethylene production, we also measured the effects of glutamate, glutamine, and proline on ethylene production. As glutamate and glutamine can be convented to AKG via oxidative deamination during the metabolism of amino

acids. We deduced that the addition of glutamate and glutamine to the culture medium may increase ethylene production by increasing intracellular AKG levels.

The strain ZM1360-*efe* which showed the comparable ethylene production among the knockout strains was used to measure the effect of substrate availability on ethylene production. Ethylene production with 5 mM proline, 5 mM arginine and 5 mM glutamic acid was 6.58 nmol /OD_{600}/mL, 10.15 nmol /OD_{600}/mL and 8.94 nmol /OD_{600}/mL. The greatest improvement in production was observed via the addition of AKG and glutamine to the growth media, with 5 mM AKG and 5 mM glutamine compared to cultures grown in standard RM media (2 % glucose): 12.8 nmol/ /OD_{600}/mL and 11.6 nmol /OD_{600}/mL (**Fig. 7**), respectively.

Ethylene production from straw enzymatic hydrolysate

In our laboratory, there is currently a strain of ZM532, which can withstand 5.0 g/L furanaldehyde and 3.0 g/L acetic acid (Unpublished data, ZM532 has been deposited at Guangdong Microbial Culture Center under the Accession Number GDMCC60527). Therefore, in order to produce ethylene form cellulosic hydrolysate, we introduced pEZ15Asp-*efe* plasmid in stress tolerant strain ZM532, and successfully obtained engineered strain ZM532-*efe.*

Cellulsoic hydrolysate obtained from pretreated straw contained 22.6 g/L glucose and 25.3 g/L xylose in this study. But its nitrogen content was only 0.9 g/L. Nutrient has great impact on sugar utilization as the ability of strains to achieve a high level of metabolite. So, we prepared RM medium with straw enzymatic hydrolysate as the sole carbon source, and tested the ethylene yield of strain ZM532-*efe*. Finally, we reached the ethylene production of 5.8 nmol/OD_{600}/ml.

Discussion

The engineering of the microbial bioethylene is a viable alternative to current ethylene production methods and represents a unique challenge. In this study, we successfully converted the non-ethylene producing strain into an ethylene producer via heterologous expression of the *efe* gene in *Z. mobilis* and reached the ethylene production of 12.8 nmol/OD_{600}/ml. Additionally, strain ZM532-*efe* obtained ethylene production of 5.8 nmol/OD_{600}/ml when we used enzymatic hydrolysis of corn straw as the sole carbon source, which was of great significance for the utilization of cellulosic biomass in the future.

A series of experiments were further conducted to investigate the factors affecting ethylene production. The results showed the ethylene production did not increase as expected with the increase of glucose concentration, the expansion of the culture volume, and the extension of the culture time which indicates that there were other factors affecting the ethylene production. Combined with the ethylene production formula 3α-ketoglutarate+Arginine+$3O_2$=$2C_2H_4$+$7CO_2$+$3H_2O$+guanidine+P5C, we concluded that O_2 is a important external impact factor in ethylene production. Furthermore, as a gaseous product, quantitative detection of the ethylene production required a rigidly sealed system. Therefore, the inability to provide a

continuous supply of oxygen (a reactant in the proposed EFE reaction) limited the availability of O_2 during the cultivation process. The restriction of O_2 maybe the key factor affecting ethylene production. This conjecture was also consistent with previous work in *E. coli* [9] and yeast [10]. Meanwhile, previously work has shown that intracellular levels of AKG reached their highest levels in early growth [33] which might be the reason for the decline in ethylene production in the culture later period.

As shown in **Fig. 6**, there are many branch metabolic pathways in the ethylene biosynthetic pathyway in wild-type strain *Z. mobilis*. And these competitive metabolic pathways are not only conducive to produce ethylene, but also generate other by-products. Previous work has shown that knockout of key enzyme genes in the ED metabolic pathway of ZM4 reverses metabolic pathway [34, 35], resulting in increased ethylene production. These analyses prompted us to design a series of genetic engineering programs to increase ethylene production. As expected, The strains ZM1360-*efe* significantly increased by 51.6 %, were 8.3 nmol/OD_{600}/ml (**Fig. 4c**). Meanwhile, compared with wild-type ZM4, PDC expression of ZM1360 in the absence of EFE decreased by 82 % [36], in other words, the down-regulation of PDC expression in strain ZM1360-*efe* led to more metabolic flow to ethylene production. This finding is consistent with previous study demonstrating that down-regulation of PDC expression could lead to the accumulation of endogenous pyruvate in *Z. mobilis* [37] and improve ethylene production. Besides, ethylene production of strain ZM1237-*efe*, ZM1570-*efe*, and ZM1596-*efe* all improved production compared with strain ZM4-*efe*. These results further demonstrate that the strategy of increasing ethylene production by knocking out these competitive metabolic pathways in ZM4 is feasible.

Additionally, the expression of *efe* at the transcriptional level was explored when OD_{600} value of five ethylene-producing strains was about 0.4. Compared with strain ZM4-*efe*, the four ethylene-producing strains did show increased expression of *efe* gene at the transcription level per cell (**Fig. 5)**. We found that although the expression of genes was consistent with ethylene production as a whole, there were still some differences (**Fig. 5 and Fig. 4c**). This difference may be related to the catalytic mechanism of EFE. Based on the equation 3α-ketoglutarate+Arginine+$3O_2$=$2C_2H_4$+$7CO_2$+$3H_2O$+guanidine+P5C for EFE-dependent ethylene production [38, 39], previous work have proposed two-loop mechanism and consider EFE as a hybrid enzyme capable of catalyzing two different reactions [39, 40]. Therefore, this may be the reason why the expression of *efe* gene and ethylene production do not correspond completely. The function and catalytic mechanism of EFE need to be further validated to provide a systematic overview to enhance understanding and guide engineering method

It should be noted that there was a 3 h lag in the cell growth, glucose utilization and ethanol production in the five ethylene-producing strains (**Fig. 4a, b and d)**.The knockout of specific genes of four strains will inevitably lead to the accumulation of NADH/NAD^+ in cells and affect the normal metabolism and cell growth. Previous work proved that the heterologous expression of *efe* from *P. syringae pv* maybe cause a depressed specific growth rate indicating a severe metablic stress [15] . These two factors may explain the slow growth of the five recombinant strains.

These genomic modifications all increase ethylene production, which highlight the importance of rational metabolic engineering. As a preferred host in cellulosic ethanol fermentation, most of the carbon flow is oriented to produce ethanol. Therefore, in order to increase ethylene production, we seek to redirect the carbon flow of the model ethanologen *Z. mobilis* to improve ethylene production. Obviously, this engineering strategy is different from that used in *E. coli* [9] and *S. cerevisiae* [10, 41, 42], but it is more propitious to *Z. mobilis*.

The metabolic pathways and substrate optimizations explored in this study are certainly not exhaustive, but they provide a good reference point for future optimization engineering work in other organisms. In addition to knocking out competing pathways, knocking out and overexpressing genes involved reactant in the proposed EFE reaction is another way to improve ethylene production. For example, previous studies have shown that knocking out *argR*, which regulates arginine biosynthesis, and *gltBD*, which regulates alpha-ketoglutarate biosynthesis, lead to respectively increase the availability of arginine and alpha-ketoglutarate, and further improve the carbon flux toward AKG[8, 24].

There are a variety of inhibitors in cellulolytic hydrolysates, such as furfural, organic acids and phenols, which will affect the normal growth and metabolism of bacterial strains and further affect the subsequent production studies [43-46]. In fact, the strain ZM532-*efe* obtained 5.8 nmol/OD_{600}/ml of ethylene production when we used enzymatic hydrolysate of corn straw as the sole carbon source, which show great potential for production of ethylene from cellulosic biomass in the future.

To summarize, we have demonstrated that heterologous expression of the *efe* genes in ZM4 can transform ZM4 into an ethylene producer, and specific targeted modification and optimization of additives can improve ethylene production to a certain extent. Finally, we further confirmed the potential of ZM532-*efe* in the production of ethylene from cellulase hydrolysate. In the next phase of our studies, we plan to explore the function and catalytic mechanism of EFE need to be further validated to provide a systematic overview to enhance understanding and guide engineering method.

Materials And Methods

Strains, plasmids and culture conditions

All strains used in this study are listed in **Table 1**. *Z. mobilis* ZM4 (ATCC 31821) was used as the wild-type strain and engineered ethylene production. Different mutant ZM4 strains ZM1360 (Δ*pdc*), ZM1570 (Δ*pfl*), ZM1596 (Δ*adhB*), and ZM1237 (Δ*ldhA*) were used for further improvement of ethylene production. The strain ZM4 and all mutant strains used in this study were static cultivated at 30℃ and cultured in rich medium (RM) containing 2.0 % glucose as a carbon source, 1.0 % (w/v) yeast extract, 0.2 % (w/v) KH_2PO_4, 0.1 % (w/v) $(NH4)_2SO_4$ and 0.2 % (w/v) $MgSO_4$ or on plates added to 1.5 % agar power. *E. coli* β10 was used for gene manipulation. It was grown in Luria-Bertani (LB) liquid medium containing 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl or on LB agar plates at 37°C for 14 h. Spectinomycin (100 μg /ml) were added in RM or LB culture media.

All DNA manipulation, including plasmid preparation from *E. coli*, restriction enzyme digestion, ligation, *E. coli* transformation and agarose gel electrophoresis were performed according to standard protocols [47]. All primers and plasmids used in this study are summarized in **Table 1**. Plasmid pEZ15Asp was used as backbone vector. A 1,050bp fragment form *P. syringae pv* (D131612) encoding the EFE was synthesized using the pUC57-Amp as the vector in the company of GENEWIZ. And then the sequence of *efe* was amplified from synthesized pUC57-*efe* using PCR with P1 primer (5'-TGAGCCTGTCGCGCGGGT-3'); P2 primer (5'-AATATATGGAGTAAGCAATGACCAACCTACAGACTTTCGA-3'), including 17 bp overlaps of *Z. mobilispdc* promotor. The strong *pdc* promotor and terminator were further amplified from the genomic DNA of ZM4 using PCR with P3, P4 primer and P5, P6 primer, respectively. Plasmid pEZ15Asp-GFP was used as template to amplify the sequence of GFP with P7, P8 primer. And subsequently four segments including *efe*, P_{pdc}, T_{pdc} and GFP ligated into linearized pEZ15Asp vector using PCR with P9, P10 primer by Gibson assembly (**Table 1**).

After transforming the ligated fragments into *E. coli* β10, tranformants were screened on LB agar plates with 100 (μg/mL) spectinomycin. After 14 h incubation at 37°C, the recombinant pEZ15Asp-*efe* plasmid was identified by PCR amplification and digestion of *EcoR*I and *Bgl*I. A recombinant plasmid pEZ15Asp-*efe* was obtained successfully and used for EFE expression. And then plasmid pEZ15Asp-*efe* was electroporated into competent cell of *Z. mobilis* (ZM4, ZM1596, ZM1570, ZM1360, ZM1237, and ZM532) using the method as described previously [48]. Tranformants were screened on RM agar plates with 100 (μg/mL) spectinomycin. After 3 days incubation at 30°C, positive clone was identified by colony PCR with P1, P2 primer and DNA sequencing. And then six strains were obtained successfully.

Measurement of ethylene production by GC-FID

Ethylene production was analyzed by Gas chromatograph (GC). A single colony from a freshly RM plate with spectinomycin resistance was transferred to 3 ml RM liquid medium and then incubated at 30℃ for 16 h. When the concentration of bacterium is around OD_{600} = 1.5, the culture was inoculated in a fresh medium at a ratio of 1:20. The inoculated fresh medium with a starting OD_{600} of 0.1-0.2 was incubated in serum bottles with rubber stoppers and aluminum cap and cultured at 30℃. The measurement of ethylene production was performed on a GC with a flame ionization detector (GC-FID) by injecting 1 ml headspace collected in serum bottles. The 20.5 ppm standrand ethylene was used for calculating the ethylene production. The Agilent GC7890A (GC-FID) was equipped with a Phas Al_2O_3 capillary column for separating the gas and operated under the following conditions: column dimension, 30m×53mm×20um; max temperature of the column, 200℃; carrier gas, Helium; oven temperature, 55℃; Front Inlet temperature, 105℃; Front Det temperature, 200℃; the flow rate of column, 6ml/min.

Analytical methods

Cell density was performed by a UV Spectrophotometer at OD_{600}, while concentrations of glucose and ethanol were determined by HPLC with a HPX-87H column. Fresh cultures were subject to incubate at 30℃. One milliliter of culture was harvested at the proper time by centrifuging at 4,500×g for 2 minutes,

and then suspensions were collected and diluted 10 times used for analyzing the glucose and ethanol by High-performance liquid chromatograph (HPLC, Agilent 1200) with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min, a column temperature of 35℃ and the injection volume at 20.0 μL.

RT-qPCR analysis The total RNA of five ethylene-producing recombinant and wild-type *Z. mobilis* strains were extracted via the Qiagen 74101 RNeasy Mini Kit and assayed according to the manufacture's instructions. And then, the obtained total RNA as the template, respectively. The first strand cDNA was synthesized by using the FastQuant cDNA kit and used to perform the expression of the *efe* of five ethylene-producing strains and *Z. mobilis* by RT-qPCR with 16s F primer (TCAACTATAGACCAGTAAGT); 16s R primer (AGAACATAGAAGAGGTAAGT) and *efe*-F primer (TACCGACAAGTCCTTGCAGA); *efe*-R primer (TCAGATCAAGACCGATAGTG). **Western blotting analysis**Total soluble proteins extracted from cell extracts of five ethylene-producing strains were separated by 12 % SDS-PAGE according to the standard procedure, and then transferred to a niticellulose. Immunoblotting was performed via Pierce™ Fast Western Blot Kit, ECL Substrate. GFP-specific antibody was prepared from the serum of a mouse by Abbkine company.

Enzymatic hydrolysis of corn straw

Thirty gram of pre-treated corn straw dried at 105℃ was added into 250 ml triangular bottle in five batches. For the first time, 10 g of corn straw, 5,600 U/g of cellulase, 94 ml of pH4.8 0.05 M sodium citrate buffer and 30 mg/L of chloramphenicol were added. After that, 5 g of corn straw and 1ml of cellulase were added in four times, each time at an interval of 4 h, and treated continuously for 144 h at 50℃ and 120 rpm. At the end of treatment, the centrifuge tube was placed in a water bath at 100℃ to inactivated the enzyme and terminate the reaction. The centrifuge tube was centrifuged at 4,500 rpm for 3 min to obtain the supernatant. Finally, the bacteria were filtered and the enzymatic hydrolysate was harvested.

Conclusion

This work first constructed an ethylene producer via heterologous expression of the *efe* gene in *Z. mobilis* and achieved stable ethylene production. In this study, methods of the metabolic pathways and substrate optimizations efficiently enhances ethylene production. Besides, strain ZM532-*efe* show great potential for production of ethylene from cellulosic biomass in the future.

Declarations

Acknowledgements

This research was financially supported by the National Natural Sciences Foundation of China (Grant No. 31570055), the Elite Program and Basic Research Program of Chinese Academy of Agricultural Sciences, and Central Public-interest Scientific Institution Basal Research Fund (No. Y2019XK23-01).

Authors' contributions

MXH designed the whole study and edited the entire draft manuscript. YH participated in all experiments and data collection. BW participated in data analysis. WX, KYZ, QY, TQ, QHY, and PTL 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.

Conflicts of interest

The authors declare that they have no competing interests.

Funding information

This research was financially supported by the National Natural Sciences Foundation of China (Grant No. 31570055), the Elite Program and Basic Research Program of Chinese Academy of Agricultural Sciences, and Central Public-interest Scientific Institution Basal Research Fund (No. Y2019XK23-01) to Mingxiong He.

Availability of supporting data

The *Z. mobilis* 532 has been deposited at Guangdong Microbial Culture Center (GDMCC) under the Accession Number GDMCC60527.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Abbreviations

EFE: the ethylene-forming enzym

RM: Rich Medium

OD: Optical Density

HPLC: High Performance Liquid Chromatography

GC: Gas Chromatography

AKG: α-ketoglutarate

References

1. Ipatieff V, Corson B. Gasoline from ethylene by catalytic polymerization. Ind Eng Chem Res. 1936;28(7):860-863.
2. Ren T, Patel MK, Blok K. Steam cracking and methane to olefins: Energy use, CO2 emissions and production costs. Energy. 2008; 33(5):817-833.
3. Hall M, Smith A. Ethylene and the responses of plants to stress. Bulg J Plant Physiol. 1995; 21(2-3):71-79.
4. Weingart H, Völksch B, Ullrich M. Comparison of ethylene production by *Pseudomonas syringae* and *Ralstonia solanacearum*. Phytopathology. 1999; 89(5):360-365.
5. Nagahama K, Ogawa T, Fujii T, Tazaki M, Tanase S, Morino Y, Fukuda H. Purification and properties of an ethylene-forming enzyme from *Pseudomonas syringae* pv. *phaseolicola* PK2. Microbiology. 1991; 137(10):2281-2286.
6. Völksch B, Weingart H. Comparison of ethylene-producing Pseudomonas syringae strains isolated from kudzu (Pueraria lobata) with *Pseudomonas syringae* pv. *phaseolicola* and *Pseudomonas syringae* pv. *glycinea*. Eur J Plant Pathol. 1997; 103(9):795-802.
7. Fukuda H, Ogawa T, Ishihara K, Fujii T, Nagahama K, Omata T, Inoue Y, Tanase S, Morino Y. Molecular cloning in *Escherichia coli*, expression, and nucleotide sequence of the gene for the ethylene-forming enzyme of *Pseudomonas syringae* pv. *phaseolicola* PK2. Biochem Biophys Res. Commun. 1992; 188(2):826-832.
8. Ishihara K, Matsuoka M, Inoue Y, Tanase S, Ogawa T, Fukuda H. Overexpression and in vitro reconstitution of the ethylene-forming enzyme from *Pseudomonas syringae*. J Ferment Bioeng. 1995; 79(3):205-211.
9. Lynch S, Eckert C, Yu J, Gill R, Maness P-C. Overcoming substrate limitations for improved production of ethylene in *E. coli*. Biotechnol Biofuels. 2016; 9(1).
10. Pirkov I, Albers E, Norbeck J, Larsson C. Ethylene production by metabolic engineering of the yeast *Saccharomyces cerevisiae*. Metab Eng. 2008; 10(5):276-280.
11. Ishihara K, Matsuoka M, Ogawa T, Fukuda H. Ethylene production using a broad-host-range plasmid in *Pseudomonas syringae* and *Pseudomonas putida*. J. Ferment. Bioeng. 1996; 82(5):509-511.
12. Tao L, Dong HJ, Chen X, Chen SF, Wang TH. Expression of ethylene-forming enzyme (EFE) of *Pseudomonas syringae* pv. *glycinea* in *Trichoderma viride*. Appl Microbiol Biotechnol. 2008; 80(4):573.
13. Chen X, Liang Y, Hua J, Tao L, Qin W, Chen S. Overexpression of bacterial ethylene-forming enzyme gene in *Trichoderma reesei* enhanced the production of ethylene. Int J Biol Sci. 2010; 6(1):96.
14. Ungerer J, Tao L, Davis M, Ghirardi M, Maness PC, Yu J. Sustained photosynthetic conversion of CO_2 to ethylene in recombinant *cyanobacterium Synechocystis* 6803. Energy Environ Sci. 2012; 5(10):8998-9006.

15. Takahama K, Matsuoka M, Nagahama K, Ogawa T. Construction and analysis of a recombinant *cyanobacterium* expressing a chromosomally inserted gene for an ethylene-forming enzyme at the psbAI locus. J Biosci Bioeng. 2003; 95(3):302-305.
16. Guerrero F, Carbonell V, Cossu M, Correddu D, Jones PR. Ethylene synthesis and regulated expression of recombinant protein in *Synechocystis* sp. PCC 6803. PloS one. 2012; 7(11):e50470.
17. Perlack RD, Wright LL, Turhollow AF, Graham RL, Stokes BJ, Erbach DC. Biomass as feedstock for a bioenergy and bioproducts industry: the technical feasibility of abBillion ton annual supply. 2005.
18. Ranatunga T, Jervis J, Helm R, McMillan J, Hatzis C. Identification of inhibitory components toxic toward *Zymomonas mobilis* CP4(pZB5) xylose fermentation. Appl Biochem Biotechnol. 1997; 67(3):185-198.
19. Gutierrez MGD, Karim MN. Influence of furfural on the recombinant *Zymomonas mobilis* strain CP4 (pZB5) for ethanol production. The Journal of American Science. 2005; 1(1):24-27.
20. Franden MA, Pienkos PT, Zhang M. Development of a high-throughput method to evaluate the impact of inhibitory compounds from lignocellulosic hydrolysates on the growth of *Zymomonas mobilis*. Journal of Biotechnology 2009, 144(4):259-267.
21. He MX, Wu B, Shui ZX, Hu QC, Wang WG, Tan FR, Tang XY, Zhu QL, Pan K, Li Q et al. Transcriptome profiling of *Zymomonas mobilis* under furfural stress. Appl Microbiol Biotechnol. 2012; 95:189-199.
22. Mills TY, Sandoval NR, Gill RT. Cellulosic hydrolysate toxicity and tolerance mechanisms in *Escherichia coli*. Biotechnol Biofuels. 2009; 2:26.
23. 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; 6:99.
24. He MX, Wu B, Qin H, Ruan ZY, Tan FR, Wang JL, Shui ZX, Dai LC, Zhu QL, Pan K et al. *Zymomonas mobilis*: a novel platform for future biorefineries. Biotechnol Biofuels. 2014; 7(1):101.
25. 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; 50:57-73.
26. Leigh D, Scopes RK, Rogers PL. A proposed pathway for sorbitol production by *Zymomonas mobilis*. Appl Microbiol Biotechnol. 1984; 20(6):413-415.
27. Widiastuti H, Lee NR, Karimi IA, Lee DY. Genome-Scale In Silico Analysis for Enhanced Production of Succinic Acid in *Zymomonas mobilis*. Processes. 2018; 6(4):30.
28. Hui. YS, Mohagheghi A, Franden MA, Chou Y, Chen X, Dowe N, Himmel ME, Zhang M. Metabolic engineering of *Zymomonas mobilis* for 2,3-butanediol production from lignocellulosic biomass sugars. Biotechnol Biofuels. 2016; 9(1):189.
29. Wang W, Wu B, Qin H, Liu P, Qin Y, Duan G, Hu G, He M. Genome shuffling enhances stress tolerance of *Zymomonas mobilis* to two inhibitors. Biotechnol Biofuels. 2019; 12(1):288.
30. He. MX, Wu. B, Shui. ZX, Hu. GQ, Wang. WG, Tan. FR, Tang. XY, Zhu. QL, Pan. K, Li. Q et al. Transcriptome profiling of *Zymomonas mobilis* under furfural stress. Appl Microbiol Biotechnol. 2012; 95(1):189-199.

31. Wu B, Qin H, Yang Y, Duan GW, Yang SH, Xin FX, Zhao C, Shao H, Wang Y, Zhu Q. Engineered *Zymomonas mobilis* tolerant to acetic acid and low pH via multiplex atmospheric and room temperature plasma mutagenesis. Biotechnol Biofuels. 2019; 12(1):10.
32. Duan G, Wu B, Qin H, Wang WT, Tan Q, Dai Y, Qin Y, Tan F, Hu GQ, He MX. Replacing water and nutrients for ethanol production by ARTP derived biogas slurry tolerant *Zymomonas mobilis* strain. Biotechnol Biofuels. 2019; 12:124
33. Wiechert W, Möllney M, Petersen S, de Graaf AA. A universal framework for 13C metabolic flux analysis. Metab Eng. 2001; 3(3):265-283.
34. Lee KY, Park JM, Kim TY, Yun H, Lee SY. The genome-scale metabolic network analysis of *Zymomonas mobilis* ZM4 explains physiological features and suggests ethanol and succinic acid production strategies. Microb Cell Fact. 2010; 9(1):94.
35. Jeong-Sun S, Chong HY, Jeong-Hyun K, Jae-Young K. Method for mass production of primary metabolites, strain for mass production of primary metabolites, and method for preparation thereof. 2007.
36. Shui ZX, Wang JL, Qin H, Wu B, Tan FR, He MX. Construction and preliminary fermentation of succinate-producing recombinant ethanologenic *Zymomonas mobilis*, Chin J. Appl Environ Biol. 2015; 21(04):657-664.
37. Zhao X. Genetic characterization and manipulation of strains of *Zymomonas mobilis* for ethanol and higher value products. Sydney: University of New South Wales; 2011.
38. Fukuda H, Fujii T, Ogawa T: Preparation of a Cell-free Ethylene-forming System from *Penicillium digitatum*. Agric Biol Chem. 1986; 50(4):977-981.
39. Fukuda H, Ogawa T, Tazaki M, Nagahama K, Fujiil T, Tanase S, Morino Y. Two reactions are simultaneously catalyzed by a single enzyme: the arginine-dependent simultaneous formation of two products, ethylene and succinate, from 2-oxoglutarate by an enzyme from *Pseudomonas syringae*. Biochem Biophys Res Commun. 1992; 188(2):483-489.
40. Hausinger RP. FeII/alpha-ketoglutarate-dependent hydroxylases and related enzymes. Crit Rev Biochem Mol Biol. 2004; 39(1):21-68.
41. Johansson N, Quehl P, Norbeck J, Larsson C: Identification of factors for improved ethylene production via the ethylene forming enzyme in chemostat cultures of *Saccharomyces cerevisiae*. Microbial cell factories 2013; 12(1):89.
42. Johansson N, Persson KO, Norbeck J, Larsson C. Expression of NADH-oxidases enhances ethylene productivity in *Saccharomyces cerevisiae* expressing the bacterial EFE. Biotechnol Bioprocess Eng. 2017; 22(2):195-199.
43. Heer D, Sauer U. Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain. Microb Biotechnol. 2008; 1(6):497-506.
44. Duque SH, Cardona CA, Moncada J. Techno-economic and environmental analysis of ethanol production from 10 agroindustrial residues in Colombia. Energy Fuels. 2015; 29(2):775-783.

45. Palmqvist E, Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresource technology 2000, 74(1):25-33.
46. Palmqvist E, Hahn-Hägerdal B: Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresour Technol. 2000; 74(1):17-24.
47. Sambrook J, E. F. Fritsch, and T. Maniatis. Molecular cloning: a laboratory manual, 2nd ed. 1989.
48. Wu B, He MX, Feng H, Zhang Y, Hu QC, Zhang YZ. Construction and Characterization of Restriction-Modification Deficient Mutants in *Zymomonas mobilis* ZM4. Chin J Appl Environ Biol. 2013; 19(02):189-197.

Table

Table 1. Cloning parts, plasmids, strains and primers used in this study.

Figures

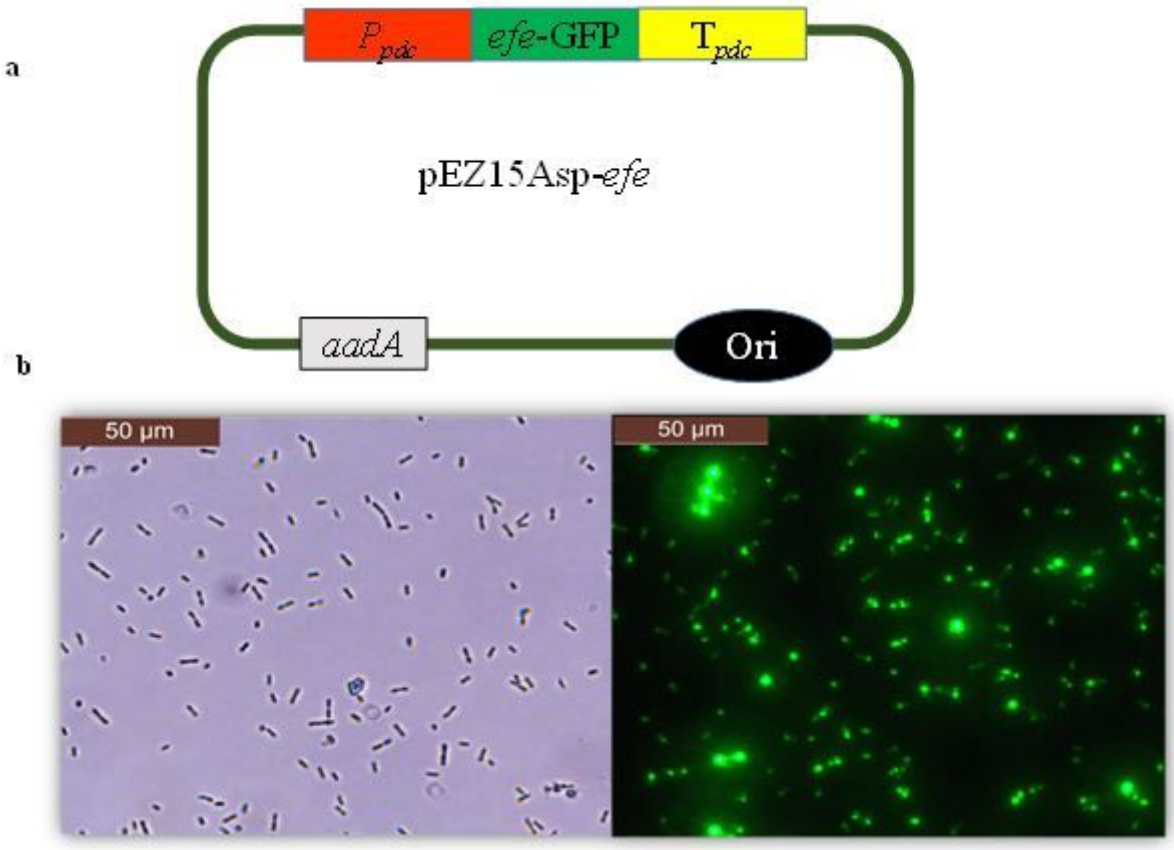

Figure 1

Constructed recombinant plasmid. a, Structural diagram of plasmid of pEZ15Asp-efe. b, expression of GFP was detected by Fluorescent Inverted microscope.

	Description	Source/remark
Strains		
Z. mobilis ZM4	Wild-type, ATCC31821	China Center of Industrial Culture collection
Z. mobilis 532		[29]
Z. mobilis strain ZM1596	ZMO1596::tet	In our laboratory
Z. mobilis strain ZM1570	ZMO1570::cm	In our laboratory
Z. mobilis strain ZM1360	ZMO1360::tet	In our laboratory
Z. mobilis strain ZM1237	ZMO1237::cm	In our laboratory
ZM4-GFP	ZM4 carrying plasmid pEZ15Asp-GFP	In our laboratory
ZM4-*efe*	ZM4 carrying plasmid pEZ15Asp-*efe*	This study
ZM1596-*efe*	ZM1596 carrying plasmid pEZ15Asp-*efe*	This study
ZM1570-*efe*	ZM1570 carrying plasmid pEZ15Asp-*efe*	This study
ZM1360-*efe*	ZM1360 carrying plasmid pEZ15Asp-*efe*	This study
ZM1237-*efe*	ZM1237 carrying plasmid pEZ15Asp-*efe*	This study
ZM532-*efe*	ZM532 carrying plasmid pEZ15Asp-*efe*	This study
Plasmids		
pEZ15Asp	Spectinomycin resistance marker	In our laboratory
pEZ15Asp-*efe*	pEZ15Asp plasmid carrying *efe* and GFP uder P*pdc* promoter and T*pdc* terminator	This study
pUC57-*efe*	pUC57-Amp plasmid carrying *efe*	This study
Primers (5’-3’)		
P1	tgagcctgtcgcgcgggt	*efe* 1050 bp
P2	aatatatggagtaagcaatgaccaacctacagactttcga	
P3	tgcttactccatatattcaaaacactatgtctg	*pdc* promotor 300 bp
P4	ttcaaggtgtcccgttccttttt	
P5	gcactgacttcaataattcagccctt	*pdc* terminator 300 bp
P6	gaattgtataaataa tttttaaataaacttagagcttaaggcg	
P7	ttatttatacaattcatccatacccaagg	GFP 720 bp

P8	cccgcgcgacaggctcaatggttagcaaaggcg	
P9	ggaacgggacaccttgaactgcaggtcaca	linearized pEZ15Asp 3009bp
P10	attattgaagtcagtgcgacgctcgagagatctgatatc	

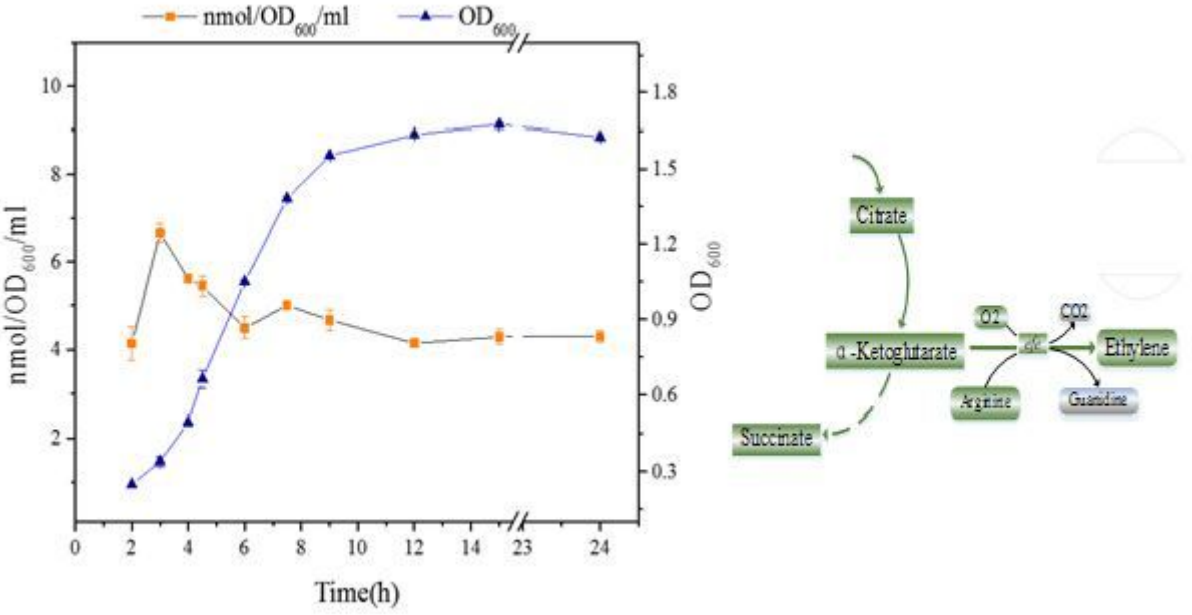

Figure 2

Ethylene production and growth as a function of time for ZM4-efe strain with EFE expressed from the pEZ15Asp-efe plasmid.

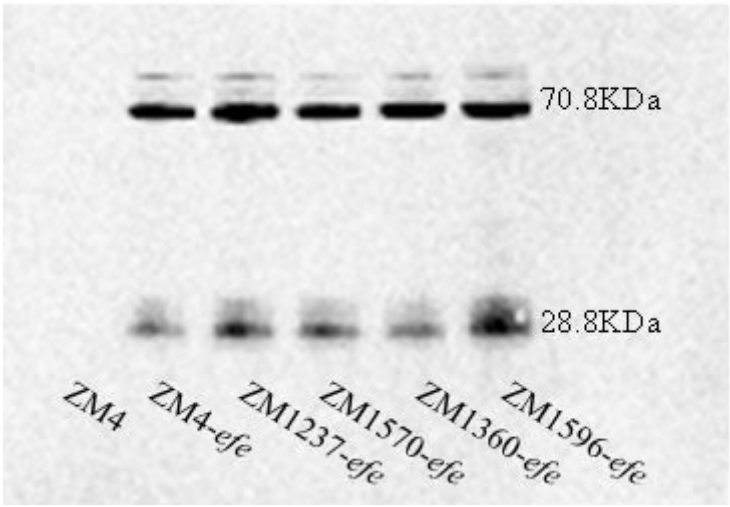

Figure 3

Western blot of GFP-Flag proteins expressed in different Z. mobilis strains

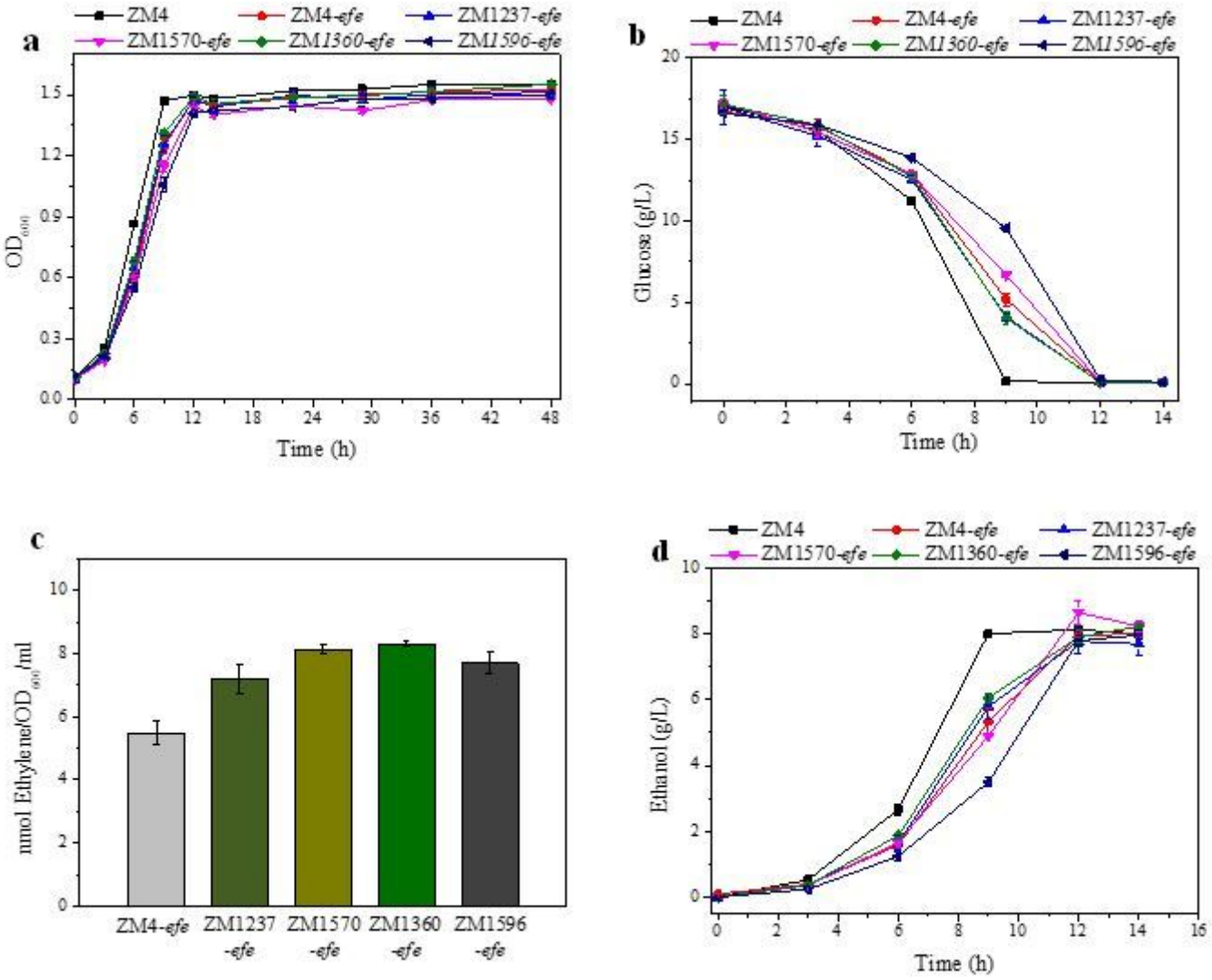

Figure 4

4 Cell growth, glucose consumption, ethylene production, and ethanol production in different Z. mobilis strains. a, Cell density as a function of time of five ethylene-producing strains and wild-type ZM4. b, Glucose utilization as a function of time of five ethylene-producing strains and wild-type ZM4. c, Peak production of ethylene of five ethylene-producing strains. d, Ethanol production as a function of time of five ethylene-producing strains and wild-type ZM4.

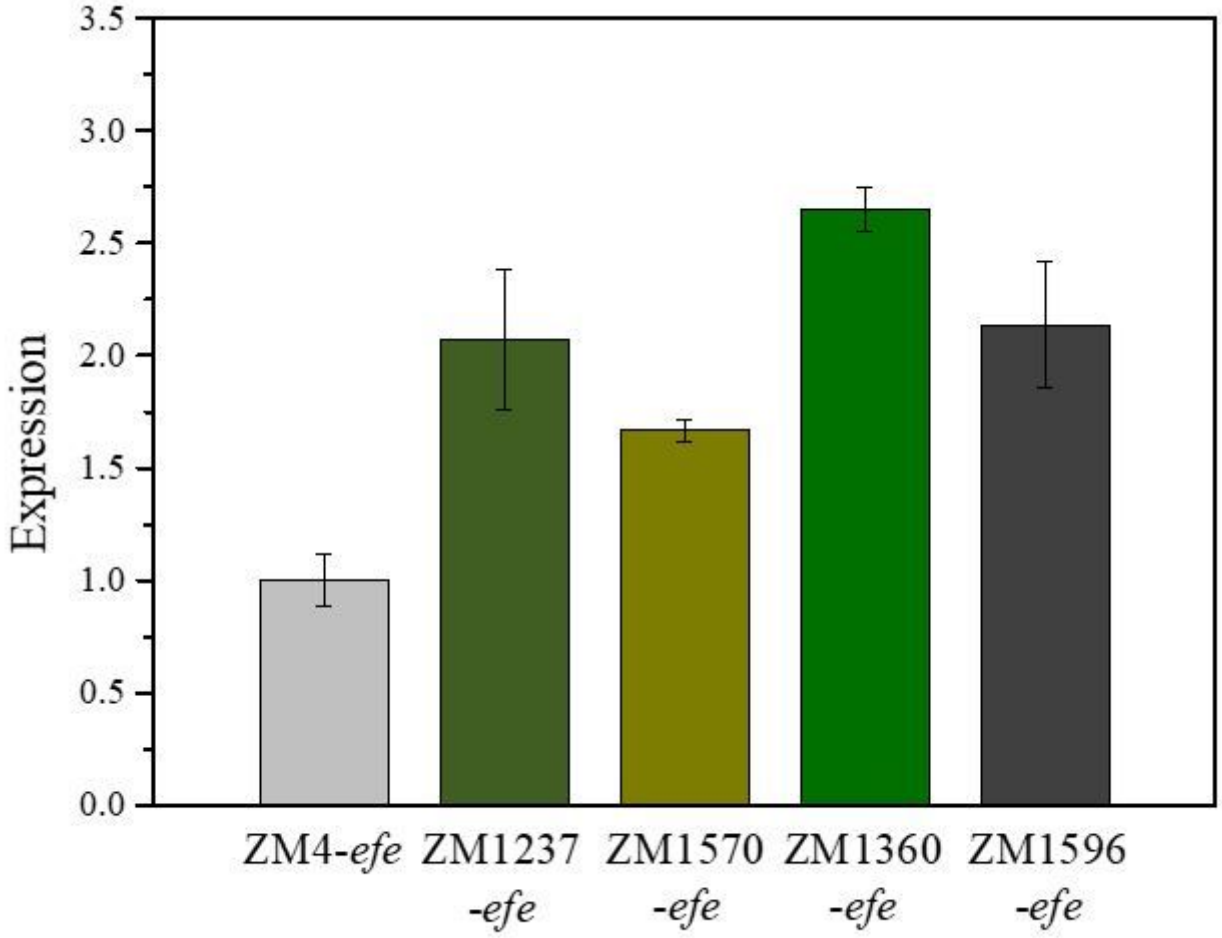

Figure 5

Expression of efe at the transcriptional level of five ethylene-producing strains Western blot of GFP-Flag proteins expressed in different Z. mobilis strains.

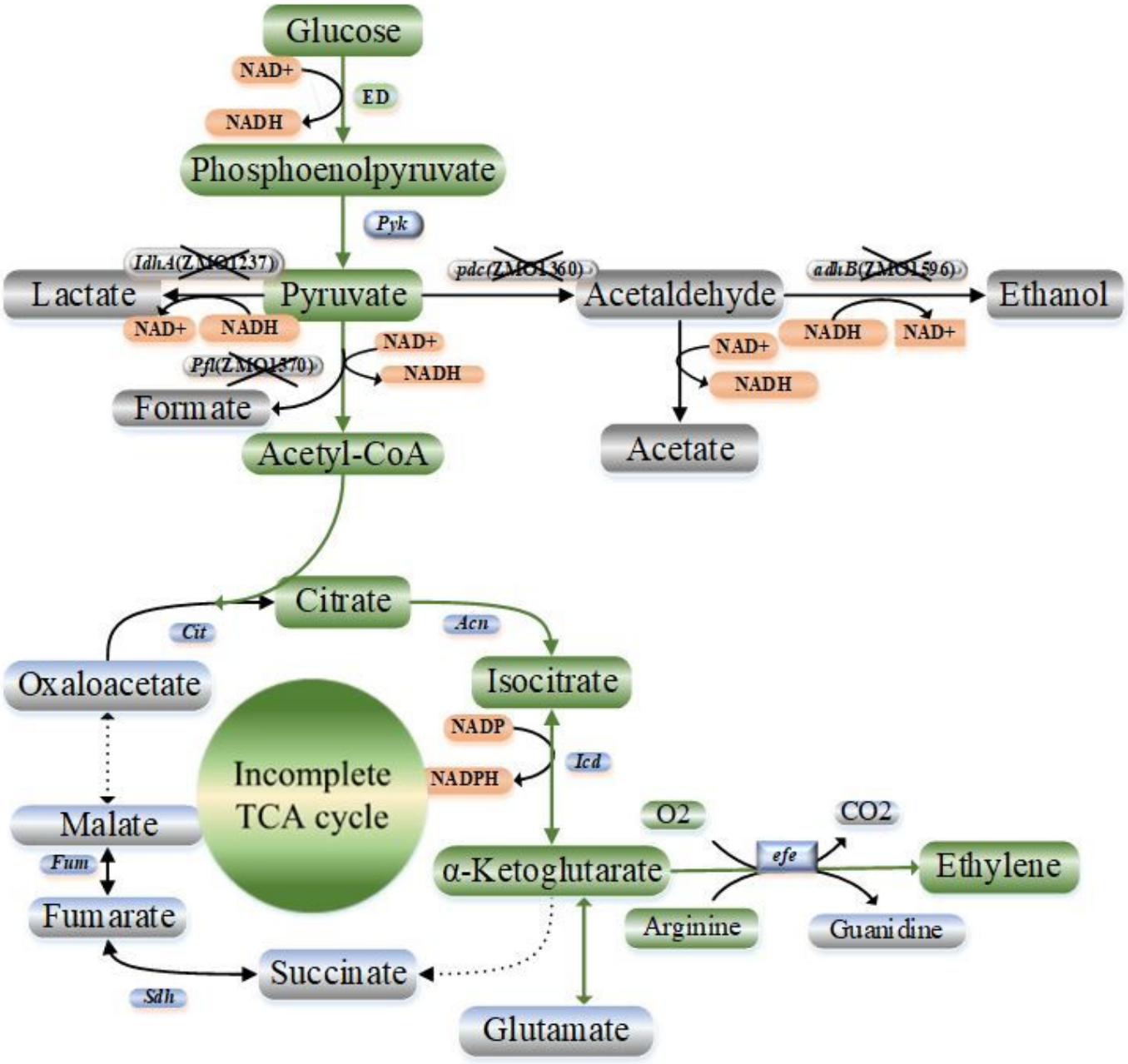

Figure 6

Biosynthetic metabolism of ethylene production in Z. mobilis by heterologous expression of efe from the Pseudomonas syringe.

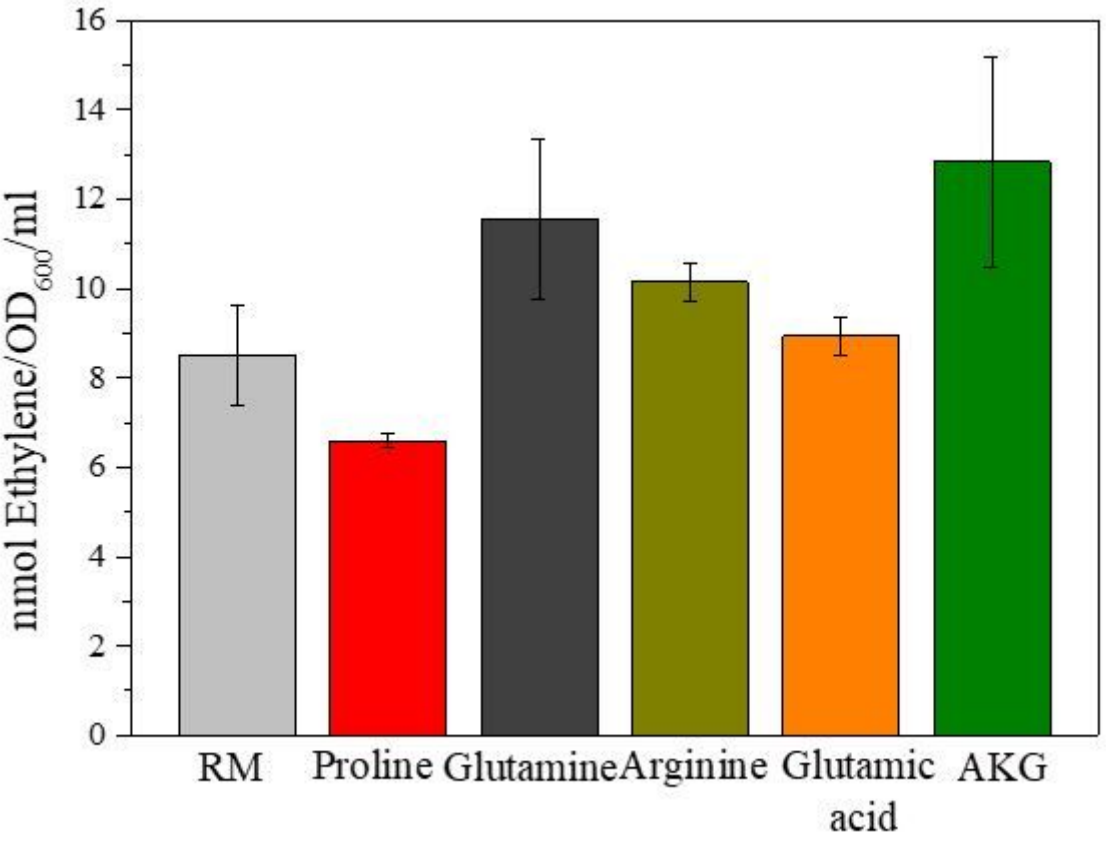

Figure 7

Production of ethylene of ZM1360-efe strain in RM media (2% glucose) supplemented with the indicated nutrient at the 5 mM concentrations.