BIOENERGY/BIOFUELS/BIOCHEMICALS

Use of an EZ-Tn5-based random mutagenesis system to create a *Zymomonas mobilis* with significant tolerance to heat stress and malnutrition

Xianghui Jia · Na Wei · Tianyv Wang · Haoyong Wang

Abstract During ethanol production, the fermentation cells are always exposed to stresses like high temperature and low nutritional conditions, which affect their growth and productivity. Stress-tolerant strains with high ethanol yield are highly desirable. Therefore, a recombinant *Zymomonas mobilis* (*Z. mobilis*) designated as *HYM* was constructed by integrating three genes (*yfdZ, metB*, and *Pfu-sHSP*) into the genome of *Z. mobilis* CP4 *(CP4)* via Tn5 transposon in the present study. The *yfdZ* and *metB* genes from *E. coli* were used to decrease the nutritional requirement. The small heat shock protein gene (*Pfu-sHSP*) from *Pyrococcus furiosus* (*P. furiosus*) was used to increase the heat tolerance. The genomic integration of three genes confers on *Z. mobilis* the ability to grow in simple chemical defined medium without the addition of amino acid. The *HYM* not only demonstrated the high tolerance to unfavorable lower nutrition stresses but also the capability of converting glucose to ethanol with high yield at higher temperature. What is more, these genetic characteristics were stable up to 100 generations on nonselective medium. The effects of glucose concentration, fermentation temperature, and initial pH on ethanol production of the mutant strain *HYM* were optimized using a Box–Behnken design (BBD) experiment. The integration of three genes led to a significant increase in ethanol production by 9 % compared with its original *Z. mobilis* counterpart. The maximum ethanol production of *HYM* was as high as 105 g/l.

Keywords Pfu-sHSP · yfdZ · metB · *Zymomonas mobilis* · Ethanol · Response surface methodology

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Introduction

Among various ethanol-producing microbes, the Gram-negative bacterium *Z. mobilis* is an efficient ethanol producer with favorable features that are at least equal to those from the more familiar brewer's yeast [9, 10]. Ethanol production at high temperature has received much attention because fermentation processes conducted at elevated temperatures will significantly reduce cooling costs and improve simultaneous saccharification, fermentation, distillation, and suitability for use in tropical countries. However, the temperatures suitable for *Z. mobilis* are relatively low (25–32 °C). *Z. mobilis* is unable to produce ethanol effectively under high temperature (above 38 °C) [2]. Creation of the heat-resistant strains will be of great value for the ethanol industry, while screens for recombinant *Z. mobilis* mutants able to produce ethanol efficiently at high temperature have never been performed before.

The hyperthermophilic archaeon *P. furiosus* expresses a small, α-crystallin-like protein in response to extreme temperatures above 103 °C. This small heat shock protein

(*Pfu-sHSP*) gives cellular protection from extremely high temperatures [7, 8] like the α-crystallin eye lens proteins, acts as molecular chaperones, and prevents aggregation of denatured proteins under heat stress. It can prevent *E. coli* cellular proteins from aggregation above 100 °C, and can significantly enhance the viability of mesophilic organisms such as *E. coli* under lethal temperatures [7]. The introduction of gene *Pfu-sHSP* might be able to protect the mesophilic enzymes of *Z. mobilis* from aggregating at elevated temperature to increase ethanol production under heat stress.

The growth of *Z. mobilis* usually requires complex rich medium containing yeast extract, and has long been known to require lysine and methionine [1, 12]. The whole-genome sequencing has revealed the specific reasons for these deficiencies. The only genes missing for lysine and methionine synthesis are *yfdZ* and *metB*, respectively [4, 6, 12]. The *yfdZ* and *metB* genes encode a PLP-dependent aminotransferase and a PLP (pyridoxal phosphate)-dependent cystathionine gamma-synthase, respectively. As the *Z. mobilis* suffered from malnutrition may not be able to maintain its normal ability to produce ethanol, which is common at the end of a batch, and rich medium will significantly increase the cost of ethanol production, it is very important to introduce *yfdZ* and *metB* genes into *Z. mobilis* to lower this organism's nutritional requirements.

Because antibiotic resistance markers are generally perceived as undesirable for industrial application, Tn5 transposition mutagenesis in *Z. mobilis* was used in this research to overcome the instability limitations of foreign plasmids exhibited under both selective and non-selective conditions. Tn5 transposition is a bacterial genetic element which can mediate genome DNA rearrangements and integration of foreign DNA via a cut-and-paste mechanism. The only macromolecular components required for this process are the transposase; the transposon, which can presumably be any sequence that is defined by two specific inverted 19-bp sequences; and the target DNA into which the insertions are made [14].

In this study, the DNA integration into the genome of *CP4* was mediated by Tn5 transposon. *Pfu-sHSP*, *yfdZ*, and *metB* genes were used for construction of a variant of *Z. mobilis* HYM having lower nutritional requirements and could be cultured in higher temperature. The optimal fermentation conditions of glucose concentration, temperature, and initial pH for maximum ethanol production of the *HYM* were quantified from the Box–Behnken design using response surface methodology. The study showed the stable integration of three genes listed above leaded to a significant improvement in the tolerance to heat stress and malnutrition stress, also led to a maximal 9 % increment in ethanol production than its original counterpart *CP4* under optimize condition.

Materials and methods

Bacterial strains and cultural conditions

The bacterial strains and plasmids used are listed in Table 1. The *E. coli* K12 MG1655 was from the American Type Culture Collection (ATCC) and was grown on Luria-Bertani (LB). *P. furious* strain was from ATCC and its genome DNA was used as template for PCR amplification of the *Pfu-sHSP* gene. *Z. mobilis* ATCC 31821 (*CP4*) was from ATCC, and was used as the template for PCR amplification of the promoter of glyceraldehyde-3-phosphate dehydrogenase (P*GAP*) and enolase (P*ENO*).

Z. mobilis strains were cultivated anaerobically in RM medium (10 g/l yeast extract, 1 g/l KH_2PO_4, 1 g/l carbamide, 0.5 g/l $MgSO_4 \cdot 6H_2O$), supplemented with different amounts of glucose (as indicated) as carbon source. For the inoculum preparation, a single colony was added to a test tube containing 5 ml RM medium and cultured anaerobically at 30 °C until it reached late exponential or early stationary phase. A 1/100 dilution was added into the pre-warmed RM medium (10 ml culture into 1,000 ml RM), which was then cultured anaerobically at 30 °C with shaking at 150 rpm for approximately 12 h, or until an OD of 2–3 at 600 nm was reached. The optical density was measured with a spectrophotometer at 600 nm. Fermentation medium and fermentation products from filter-sterilized cell-free spent medium were analyzed by gas chromatography for ethanol determinations. The reducing glucose concentration was determined using the DNS method [3, 13].

Construction of Tn5 transposon vector and transposome preparation

Promoter P*GAP/NEO* was a fusion of two strong constitutive promoters of *Z. mobilis*, glyceraldehyde-3-phosphate dehydrogenase promoter (P*GAP*) and 2-phosphoglycerate dehydratase promoter (P*NEO*) [1, 2]. Promoter P*GAP*

Table 1 Bacterial strains and plasmids used in this study

Strains	Containing genes	Source
E. coli K12 MG1655	*yfdZ*, *metB*	ATCC 47076
P. furious	*Pfu-Shsp*	ATCC 43587
Z. mobilis CP4(*CP4*)	Wild-type *Z. mobilis*	ATCC 31821
Z. mobilis HYM (*HYM*)	*yfdZ*, *metB*, *Pfu-Shsp*	This study
Plasmids		
pTN-HMY	*Pfu-sHSP*, *metB*, *yfdZ*	This study

yfdZ and *metB* genes were used for self-synthesizing lysine and methionine

Pfu-sHSP gene was used for increasing the resistance to heat stresses

DNA fragment was amplified from *CP4* chromosomal with the primer pair: 5′ ctcgagGTTCGATCAACAACCCGA and 5′ ggatccCTAACTTATTAAGTAGCTA; promoter P*NEO* was amplified with the primer pair: 5′ agatctCTC CAGTTACTCAATACG and 5′ gaattcAACCTTTCTT AAAATCTT. After the P*GAP* DNA was digested with *Bam*HI and the P*NEO* DNA was digested with *Bgl*II, two DNA fragments were mixed, and incubated with T4 DNA ligase for an additional 12 h at 18 °C to form the fusion P*GAP/NEO* Promoter. The fusion promoter was PCR amplified with primer: 5′ ctcgagGTTCGATCAACAAC CCGA and 5′ gaattcAACCTTTCTTAAAATCTT.

The *yfdZ* gene was amplified from *E. coli* K-12 chromosomal with the primer pair: 5′ gaattcATGGCTGAC ACTCGCCCT and 5′ gtcgacTTATTCCGCGTTTTCGTG. After the digestion by *Eco*RI, this 1.3-kb DNA fragment was ligated with the *Eco*RI fragment of P*GAP/NEO* to form the *Z. mobilis* expressible P*GAP/NEO-yfdZ* operon. The *metB* gene was amplified from *E. coli* K-12 chromosomal with the primer pair: 5′gaattcATGACGCGTAAA CAGGC and 5′ gtcgacTTAC CCCTTGTTTGCAGC. After the digestion by *Eco*RI, this 1.17-kb DNA fragment was ligated with the *Eco*RI fragment of P*GAP/NEO* to form the *Z. mobilis* expressible P*GAP/NEO-metB* operon. The *Pfu-sHSP* gene was amplified from *P. furious* chromosomal with the primer pair: 5′gaattcATGGTGAGGAGAATAA GA and 5′gtcgacTTATTCAACTTTAACTTC. After the digestion by *Eco*RI, this 0.5-kb DNA fragment was ligated with the *Eco*RI fragment of P*GAP/NEO* to form the P*GAP/NEO-sHSP* operon. After the P*GAP/NEO-metB* operon was digested with *Sal*I and the P*GAP/NEO_yfdZ* operon was digested with *Xho*I, two DNA fragments were ligated to get the P*GAP/NEO-metB/yfdZ* operon. After P*GAP/NEO-metB/yfdZ* operon was digested with *Xho*I and P*GAP/NEO-sHSP* operon was digested with *Sal*I, two DNA fragments were ligated to result in the P*GAP/NEO-sHSP/metB/yfdZ* operon, which could be cut out by *Sal*I and *Xho*I.

The commercial transposon EZ-Tn5 pMOD vector was modified by substituting the *Pvu*I and *Psh*AI restriction sites outside of both ends flanking Tn5 transposase recognition sequences with *Sfi*I restriction site. The resulting plasmid was named as pTN, was a high-copy number, pUC19-based vector with a multiple cloning site (MCS) between the hyperactive 19-bp Mosaic Ends (ME) that was specifically and uniquely recognized by Tn5 Transposase. The Tn5-carrying plasmid pTN was modified further for integration of foreign DNA into *Z. mobilis*. The tetracycline resistance gene mutated without *Sal*I restriction site (the 1.4-kb *Bam*HI fragment from p34S-Tc, GenBank: AF062082) was inserted into the *Bam*HI site of plasmid pTN to result in the plasmid pTN-TC. The P*GAP/NEO-sHSP/metB/yfdZ* operon was obtained by digestion with *Sal*I and *Xho*I, purified from an agarose gel and DNA

concentration was quantified, and then inserted into the *Sal*I site of pTN-TC. The resulting plasmid, designated as pTN-HMY Fig. 1), was then transformed into chemically competent *E. coli Top10* cells (Invitrogen, Carlsbad, CA, USA) and inoculated on LB agar plates supplemented with tetracycline and ampicillin and sequence confirmed.

Transposome electroporation into *CP4*

To prepare the transposome, the Tn5 transposon pTN-HYM was digested with *Sfi*I, purified from the agarose gel, and mixed with EZ-Tn5 transposase (Epicentre). The mixture was incubated for 30 min at room temperature to allow stable binding of Tn5 transposase to the Transposon DNA; that mixture was then stored at −20 °C.

After determining that *Z. mobilis* CP4 was indeed sensitive to tetracycline, fresh electrocompetent cells of *CP4* from 400 ml RM medium with an optical density at 600 nm (OD600) as 0.4, were washed twice with equal volumes of ice-cold 10 % glycerol. Cells were suspended again in 400 μl of washing buffer, and aliquots of 100 μl were dispensed into 0.2-mm gapped electroporation cuvettes along with 1 μl of pTN-HYM-Tn5 mixture. Electroporation was performed with a Bio-Rad Gene Pulser Xcell (6.0 ms, 1.5 kV) (Bio-Rad, Hercules, CA, USA). Cells were immediately diluted into 1 ml RM medium and plated onto RM agar supplemented with 30 μg/ml tetracycline.

Isolation and identification

After 2 days of incubation at 30 °C, colonies on tetracycline-containing plates arose only when the electroporation mixtures contained pTN-HYM; not a single colony was observed in the mixtures without the DNA. A total of about 5×10^2 Tc-resistant transformants were obtained in each of three independent transformation experiments from three independent transformations. We picked 100 random colonies and those cells were inoculated in fresh RM liquid medium. To verify the DNA integration, DNA was isolated using the Qiagen DNeasy blood and tissue kit, and Tn5 insertion within the genome were analyzed by PCR. After 2 mg of the isolated DNA sample was digested overnight with *Eco*RI or *Bam*HI, the digested DNA samples were electrophoresed on a conventional 0.7 % agarose gel and then transferred onto nylon membranes. After hybridization with the *metB*-specific probe (ATGACGCGTAAACAGGC), Southern blots were developed.

Single mutant colonies were cultured on the selection RM medium contains 230 g/l glucose for 3 days. Strains with the higher ethanol yield were selected for further fermentation research and kept in RM medium with 20 % glycerol at −80 °C. After the selection, one strain, with a higher ethanol yield than its original counterpart *CP4*,

Fig. 1 Constructions of Tn5 transposon plasmid pTN-HMY containing a multicistronic operon P*GAP/NEO-sHSP/metB/yfdZ*. A fusion promoter (fusion of two strong constitutive promoters: P*GAP* and P*NEO* from *Z. mobilis*) was cloned to facilitate the higher level expression of genes under its control. The *YfdZ* and *metB* genes were used to decrease nutritional requirements. The *Pfu-sHSP* gene was used to increase resistance to heat stresses. The tetracycline resistance determinant (TC) was used to allow selection of *Z. mobilis* transformants after electroporation with the Tn5 transposon pTN-HYM. This plasmid contains the mosaic end (ME) sites, which were specifically recognized by the EZ-Tn5 transposase
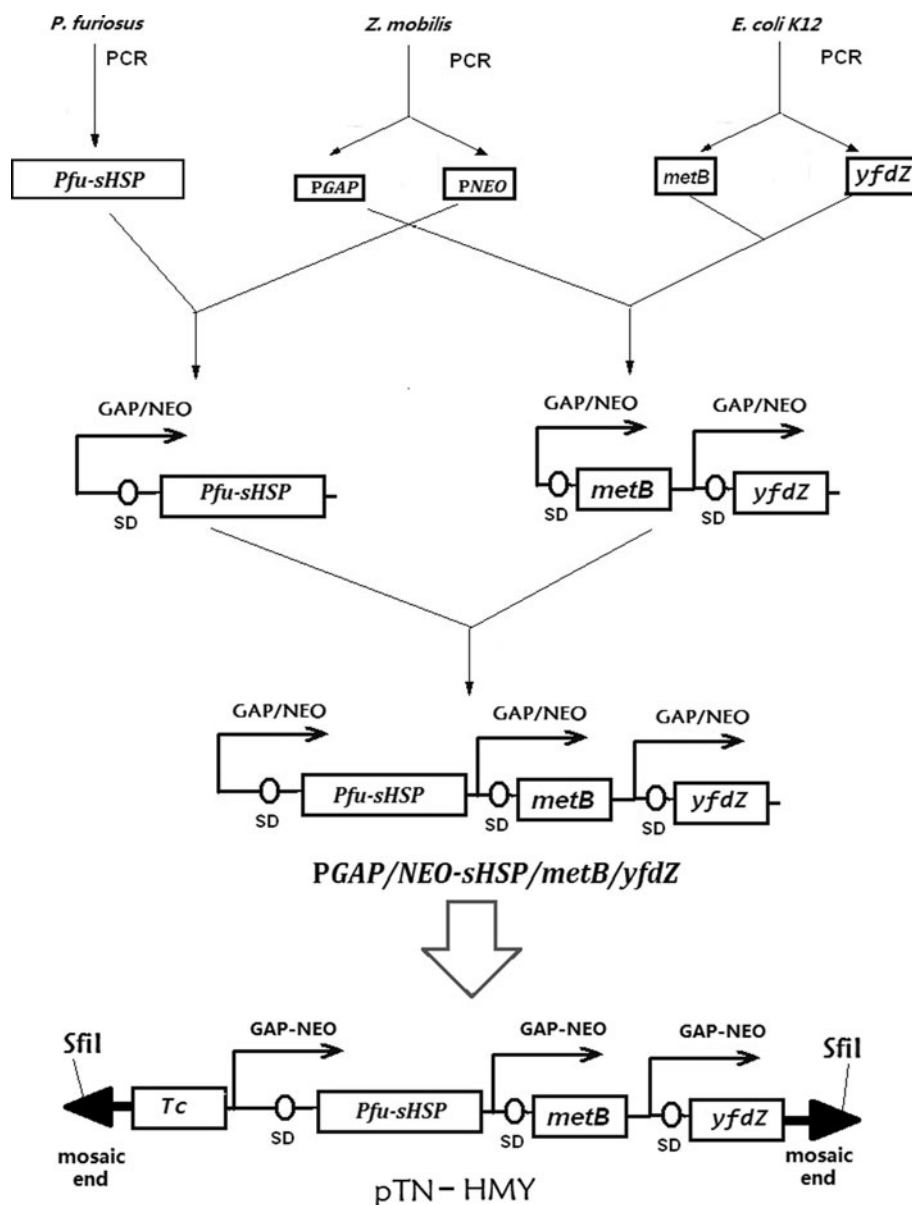
named *HYM*, was selected. All strains were fermented in the same conditions in triplicate.

Gas chromatography (GC)

Ethanol concentrations in the medium supernatant were determined by flame ionization gas chromatography. Culture samples (1 ml) and standards were prepared by filtration. The samples and standards were quantified by injecting 1 μl of each into an Agilent Technologies model 6890 equipped with a DB-FFAP 30 m × 0.53 mm × 1.5 μm film thickness capillary column (Agilent, Santa Clara, CA, USA). The column operated with an initial temperature of 80 °C and ramping 10 °C to a final temperature of 180 °C, while the detector was at 250 °C and the injector temperature was 200 °C with a post-injection dwell-time of 1 min. The carrier gas was N_2 at a constant flow rate of 5 ml/min.

Experimental design for optimization of ethanol production

To determine the principal factors during the ethanol fermentation process, the *HYM* was cultured in different fermentation temperatures (25, 30, 35, 40, and 45 °C, respectively), fermentation time (24, 48, 72, 96, 120 h, respectively), initial pH (4.0, 5.0, 6.0, 7.0, 9.0, respectively), glucose concentration (50, 100, 150, 250, 350 g/l, respectively), magnesium ions ($MgSO_4\cdot6H_2O$) concentration (0, 0.5, 1.0, 1.5, 2.0 g/l, respectively), urea

concentration (0, 0.3, 0.6, 0.9, and 1.2 g/l, respectively). Box–Behnken design (BBD) was used for the optimization of ethanol production. Temperature (X_1, °C), glucose concentration (X_2, g/l), and initial pH (X_3) were chosen as the independent variables. Ethanol concentration (Yi, g/l) was used as the dependent output variable. For statistical calculations, the variables X were coded as x_i according to Eq. (1):

$$\frac{X_i - \overline{x_i}}{\Delta x_j}, \; i = 1, 2, 3, \ldots, k \tag{1}$$

where x_i was dimensionless value of an independent variable, X_i was the real value of an independent variable, $\overline{x_i}$ was the real value of the independent variable at the center point and Δx_j was the step change.

The Box–Behnken design with a total number of 15 experiments was employed for the optimization of the parameters. The three levels of each variable were coded as -1, 0, and $+1$, which corresponded to the lower, middle, and higher values, respectively. The second-degree polynomials (Eq. 2) were calculated with the statistical package (Stat-Ease Inc., Minneapolis, MN, USA) to estimate the response of the dependent variable:

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 \\ + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \tag{2}$$

where Y_i was the predicted response, X_1, X_2, X_3 the independent variables, b_0 the offset term, b_1, b_2, b_3 the linear effects, b_{11}, b_{22}, b_{33} the squared effects, and b_{12}, b_{23}, b_{13} were interaction terms.

For a verification experiment, the optimized factors were used for the fermentation of ethanol production of HYM to confirm the accuracy of the model for predicting the maximum ethanol production. Additional experiments in sextuplicate using these optimized factors were performed.

Results

Random insertion of PGAP/NEO-sHSP/metB/yfdZ operon

Primers used for amplification of Pfu-sHSP, yfdZ, and metB genes were chosen to verify the existence of the PGAP/NEO-sHSP/metB/yfdZ operon within the mutant Z. mobilis. Confirmed by PCR reactions, 78 (78 %) of 100 strains from the RM plates containing tetracycline carried the PGAP/NEO-sHSP/metB/yfdZ operon, which was not naturally encoded by CP4. To demonstrate the usefulness of the new operon on the production of ethanol, 78 PCR-positive strains were cultured in YM medium with 230 g/l glucose as carbon source under anaerobic conditions at 32 °C with the original strain CP4 as a control. After 60-h fermentation, 65 (about 83 %) of 78 strains produce more ethanol than CP4 (data not shown). Five strains from the 65 strains with higher ethanol productivity than CP4 were chosen by random to assess the "randomness" of transposon insertion, via southern hybridization identification with a metB probe. The Southern-blot analyses suggested that the PGAP/NEO-sHSP/metB/yfdZ operon was apparently inserted into different DNA regions in these mutants, and only a single copy of the metB gene was detected in these transposon mutants (Fig. 2).

As the majority of the PCR-positive mutants produced more ethanol than CP4, it is hard to say that the insertion of the PGAP/NEO-sHSP/metB/yfdZ operon was not responsible for the improved ethanol production, and because the Z. mobilis with insertion of the operon showed varied ethanol production capability, the improvement was affected by the integration site.

Z. mobilis HYM could grow in simple defined chemical medium

The growth of Z. mobilis has long been known to need the addition of nutrition. Although it possesses a complete set of genes for synthesis of all amino acids but does not have one gene in the lysine (yfdZ) pathway and one gene in the methionine (metB) pathway. To demonstrate the effects of the Tn5 system on the nutritional requirements, one PCR-positive strain HYM was chosen to be cultured in different plates under anaerobic conditions with the original strain CP4 as a control.

There were four types of agar plates in this research. The agar plate provided a solid medium on which Z. mobilis was cultured. Liquid YM medium was mixed with agar and poured into Petri dishes to form the agar plate YM. Plate I was derived from the plate YM without yeast extract. Plate II was derived from plate I by adding various salts (NaCl, CaCl, KCl), which was a common substance for Z. mobilis growth to substitute the yeast extract, and by adding NH_4Cl to substitute carbamide. Plate III was derived from plate II with carbamide replaced by NH_4Cl. An important distinction between the four growth mediums was that plate YM was the only one with yeast extract. Plates I, II, and III were classified as defined medium with known quantities of all ingredients, while the plate YM as undefined medium with yeast extract, which consists of a mixture of many chemical species in unknown proportions.

CP4 grew well in the medium containing yeast extracts, but did not grow in simple chemical medium. With the integration of yfdZ and metB genes, the HYM strain showed normal growth both at 32 and 42 °C in simple chemical medium (Fig. 3).

Fig. 2 Southern-blot analyses of *Z. mobilis* mutants obtained after electroporation with EZ-Tn5 transposome. After selection on RM plates containing tetracycline (15 mg/ml), five strains were chosen by random for further verification. Following overnight digestion of the purified DNA with *Eco*RI or *Bam*HI, the digested DNA samples were electrophoresed on a conventional 0.7 % agarose gel. The separated DNA was then transferred onto a nylon membranes for hybridization with a Dig-labeled *metB* probe. The Southern blots were developed using reagents from the DIG DNA labeling and detection kit (Roche) according to the manufacturer's instructions (the size of the DNA fragments, in kilobases (kb), is shown in the *middle*)
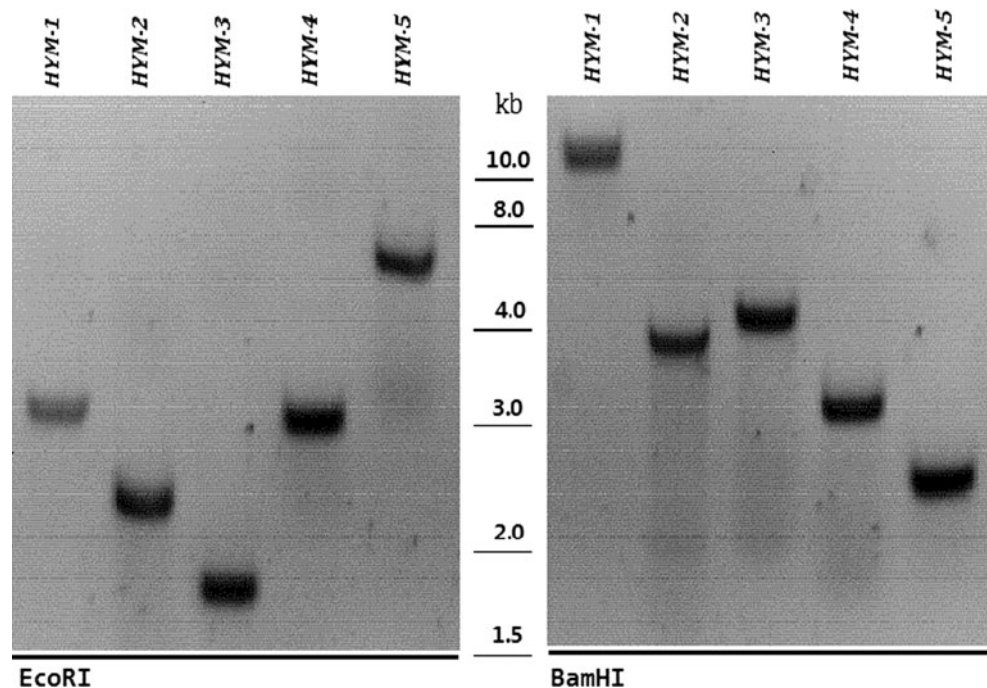
After preliminary evaluation, one strain with the highest ethanol capacity from 65 PCR-positive strains, designated as *HYM*, was selected for further fermentation studies with *CP4* as the control. When cultured in RM medium (10 g/l yeast extract, 1 g KH_2PO_4, 1 g/l Carbamide, 0.5 g/l $MgSO_4 \cdot 6H_2O$) with 230 g/l glucose as carbon source under anaerobic conditions at 32 °C for 60 h, the highest ethanol concentration of *CP4* and *HYM* was 78.4, 87.1 g/l, respectively (Fig. 4).

The effect of yeast extract concentration on ethanol production was studied in this research. According to Fig. 5, there was no obvious difference of ethanol yield between two strains in low nutrient medium. However, *HYM* was more effective in producing ethanol when the yeast extraction concentration was higher than 4 g/l. The ethanol concentration of *HYM* and *CP4* was increased by 54.5 and 39.6 g/l, respectively, when yeast extraction concentrations were changed from 4 to 8 g/l. Although strain *HYM* showed normal growth both at 32 and 42 °C in simple defined chemical medium, and could produce much more ethanol in RM medium when the yeast extraction was more than 4 g/l, yeast extract was still needed for high-level production of ethanol (Fig. 5).

Significant increase in ethanol yield and productivity at high temperature

Pfu-sHSP is an α-crystallin homologue and can act as a molecular chaperone to prevent membrane destabilization or aggregation of denatured proteins under heat and other stresses. It can prevent mesophilic enzymes from aggregation at extremely high temperatures, such as can prevent *E. coli* cellular proteins from aggregation above 100 °C, and could therefore potentially be used to maintain the viability of *CP4* under unfavorable high-temperature conditions. In the RM medium (10 g/l yeast extract, 1 g KH_2PO_4, 1 g/l carbamide, 0.5 g/l $MgSO_4 \cdot 6H_2O$) containing 230 g/l glucose after 60-h fermentation, although the ethanol production declined dramatically when the temperature reached 40 °C from 32 °C, the *Pfu-sHSP* containing bacteria *HYM* showed a significantly more heat tolerance compared with *CP4* (Fig. 6).

Stability test

HYM strain was selected for further stability testing. Frozen stock culture was transferred into RM medium (10 g/l yeast extract, 1 g KH_2PO_4, 1 g/l carbamide, 0.5 g/l $MgSO_4 \cdot 6H_2O$) with 230 g/l glucose (RMG). Overnight-grown culture was transferred into an RMG tube containing 10 ml of medium to an initial OD of 0.02 at 600 nm. At the end of 100 generations, the grown culture was also tested for fermentation of glucose in a 100-ml shake flask containing 80 ml of RMG. The strain could ferment glucose both rapidly and efficiently with no obvious difference then they were first been isolated. The PCR reaction also confirmed that the *HYM* strain was stable up to 100 generations on non-selective medium.
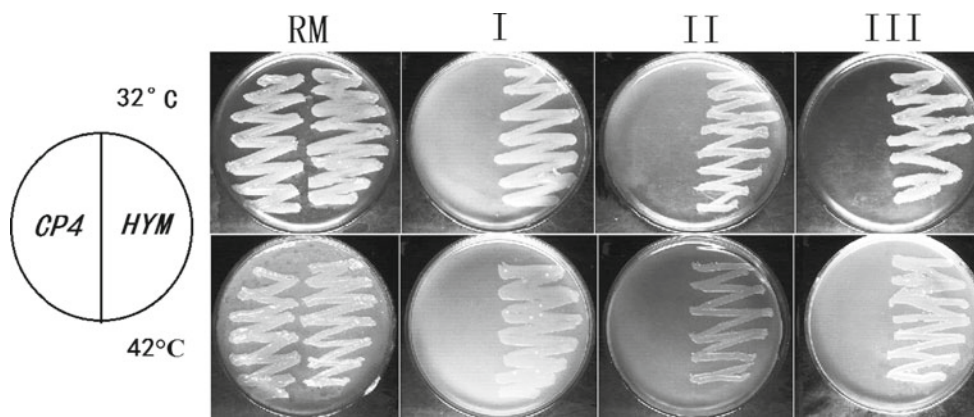
Different raw materials used for creation of experimental plate (g/L)

Media name	RM	I	II	III
Yeast extract	10.0	0	0	0
KH_2PO_4	1.0	1.0	1.0	1.0
$MgSO_4·6H_2O$	1.0	0	0	0
Carbamide	1.0	1.0	0	1.0
Glucose	10.0	10.0	10.0	10.0
NH_4Cl	0	0	10.0	0
$MgCl_2·6H_2O$	0	0	1.0	1.0
NaCl	0	0	8.0	8.0
$CaCl_2·.2H_2O$	0	0	0.4	0.4
KCl	0	0	1.0	1.0
Agar	20.0	20.0	20.0	20.0

Fig. 3 Growth properties of *CP4* and *HYM* on different media at 32 °C (*upper*) or 42 °C (*lower*) anaerobically for 3 days. *CP4* grows only in media containing yeast extracts (RM), and cannot grow in simple chemical defined media (I, II, III). *HYM* grows well in simple chemical media without yeast extract (I, II, III)

Effects of the single-factor experiments on ethanol production

The effects of fermentation temperature, fermentation time, initial pH, glucose concentration, magnesium ion concentration, and urea concentration on ethanol production of *HYM* were tested in this research. As shown in Fig. 7a, the highest ethanol production was observed at 30 °C, with ethanol production decreasing as the fermentation temperature increased. The ethanol production increased with increasing fermentation time up to 72 h but then decreased slightly (Fig. 7b). For the initial pH, the maximum ethanol production occurred at pH7 (Fig. 7c). Figure 7d shows that the initial glucose concentration of the fermentation for maximum ethanol production was at about 250 (g/l). The addition of magnesium ions and urea had neither a stimulatory nor inhibitory effect on the ethanol production (Fig. 7e, f).

Single-factor analysis of variance

The results of variance analysis for single-factor experiments for *HYM* were: the fermentation temperature, initial pH, and glucose concentration were all significant to the ethanol production of *HYM*. Other factors like magnesium ion concentration and urea concentration did not have a significant influence on ethanol production.

Optimization experiments design

Temperature, glucose concentration, and initial pH were chosen as important process parameters to optimize the fermentation conditions of *HYM* for ethanol production based on the single-factor experiments. A total of 15 experiments with different combinations of temperature, glucose concentration, and initial pH were performed (Table 2) by using the Box-Behnken design. The
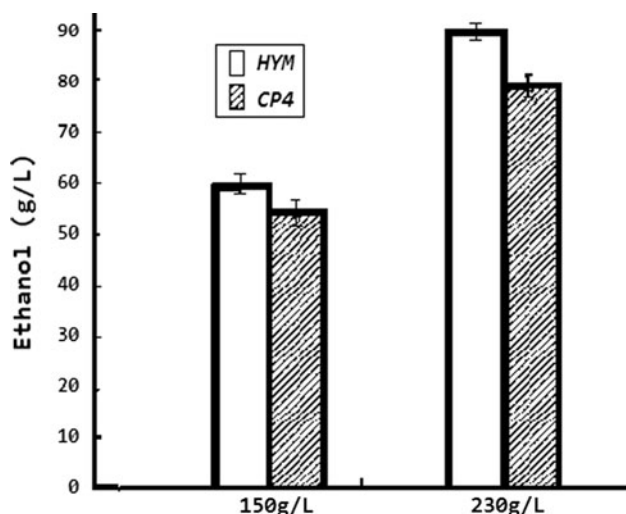
Fig. 4 Comparisons of ethanol production with different concentrations of glucose between *CP4* and *HYM*. Cultures were grown in RM medium (10 g/l yeast extract, 1 g KH$_2$PO$_4$, 1 g/l carbamide, 0.5 g/l MgSO$_4$·6H$_2$O) with 230 g/l glucose as the carbon source under anaerobic conditions at 32 °C. All strains were tested by three parallel experiments. After 60-h fermentation, the highest ethanol concentration of *HYM* was 87.1 g/l with the ethanol yield as 0.38 g/g (ethanol/glucose); while the original strain *CP4* was 78.4 g/l with the ethanol yield as 0.34 g/g (ethanol/glucose). The difference of ethanol yield between *HYM* and *CP4* was 11 %
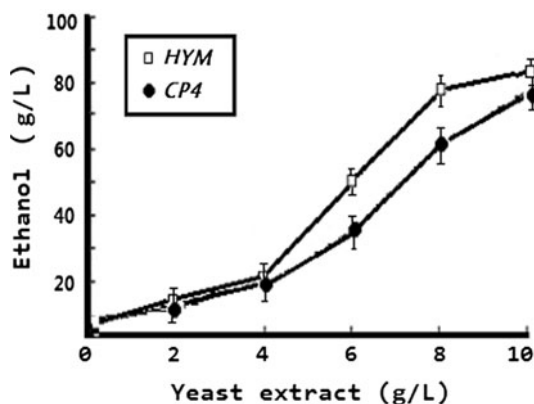

Fig. 5 Comparisons of ethanol production between *CP4* and *HYM* in media containing different concentration of yeast extract. Cultures were grown in RM medium without yeast extract (1 g KH$_2$PO$_4$, 1 g/l carbamide, 0.5 g/l MgSO$_4$·6H$_2$O) with 23 % glucose as the carbon source under anaerobic conditions at 32 °C for 60 h, supplemented with different amounts of yeast extract as indicated. All strains were tested by three parallel experiments. The experiment data proved that the yeast extract was still needed for high-level ethanol production, although *HYM* could grow without yeast extract. However, from the trend lines, we can deduced that in nutrient medium, *HYM* was more efficient in producing ethanol than *CP4*

experimental results of the BBD were fitted with a second-order polynomial equation. The values of regression coefficient were calculated and the fitting equations (in terms of coded values) for predicting the ethanol production (Y_i) was.
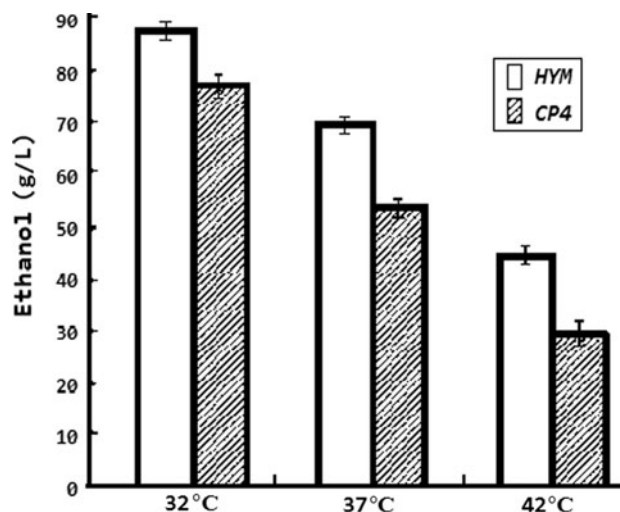
Fig. 6 Comparisons of ethanol production between *CP4* and *HYM* at different temperature. Strains were grown in RM medium (10 g/l yeast extract, 1 g KH$_2$PO$_4$, 1 g/l carbamide, 0.5 g/l MgSO$_4$·6H$_2$O) with 23 % glucose as the carbon source under anaerobic conditions. All strains were tested by three parallel experiments. When the temperature reached 42 °C from 32 °C, after 60-h fermentation, the average ethanol concentrations of *CP4* and *HYM* were reduced dramatically. When the temperature was 42 °C, the ethanol yield of *HYM* based on consumed glucose was 0.19 g/g (ethanol/glucose), significantly different from *CP4* (ethanol yield based on consumed glucose was 0.12 g/g). It is apparent that *HYM* was much less sensitive to high temperatures than *CP4*

$$
\begin{aligned}
Y_i =\ & 100.2017 - 6.675375X_1 - 7.14X_2 \\
& + 15.15563X_3 - 7.796833X_1^2 + 0.37475X_1X_2 \\
& + 1.767X_1X_3 - 30.53158X_2^2 + 19.92225X_2X_3 \\
& - 18.18633X_3^2
\end{aligned}
$$

(3)

where X_1 was temperature, X_2 was glucose concentration, and X_3 was initial pH.

The predicted levels of ethanol production using the above equation were given in Table 2. The predicted optimum levels of temperature, glucose concentration, and initial pH were obtained by applying regression analysis to the equation. The predicted and experimental ethanol productions at the optimum levels of fermentation conditions were also determined by using Eq. (3). The maximum ethanol concentration of 104.5 g/l appeared when temperature, glucose concentration, and initial pH were 28.1 °C, 241 g/l, and pH 7.4, respectively.

Response surface analysis

Figure 7 represents the isoresponse contour and surface plots for the optimization of fermentation conditions of ethanol production. The effects of the initial glucose concentration and initial pH on the ethanol production are shown in Fig. 7a. As the initial glucose concentration

Fig. 7 Effect of the single-factor experiments on the ethanol production of *HYM*. **a** Effect of fermentation temperature on ethanol production. **b** Effect of fermentation time on ethanol production. **c** Effect of initial pH on the ethanol production. **d** Effect of initial glucose concentration on the ethanol production. **e** Effect of initial magnesium ions concentration on the ethanol production. **f** Effect of initial urea concentration on ethanol production
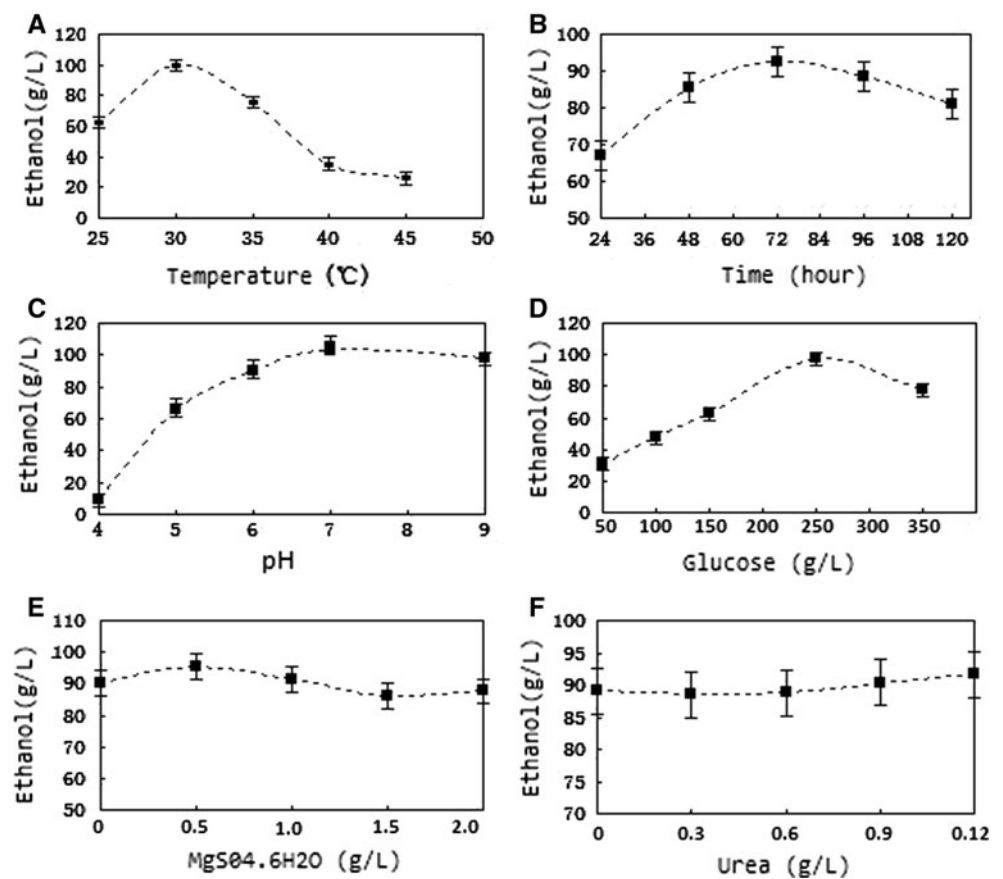

Table 2 The Box-Behnken center design matrix for the experimental design and predicted results for ethanol production

Run no.	X_1	X_2	X_3	Ethanol concentration (g/l)	
				Experimental	Predicted
1	−1	−1	0	69.631	76.06341
2	−1	1	0	58.323	61.03391
3	1	−1	0	64.674	61.96316
4	1	1	0	54.865	48.43266
5	0	−1	−1	64.240	63.39041
6	0	−1	1	56.729	53.85717
7	0	1	−1	6.394	9.26591
8	0	1	1	78.572	39.57717
9	−1	0	−1	73.088	67.50528
10	1	0	−1	47.060	50.62053
11	−1	0	1	97.843	94.28254
12	1	0	1	78.883	84.46579
13	0	0	0	100.354	100.2017
14	0	0	0	99.962	100.2017
15	0	0	0	100.289	100.2017

trend. The interaction effect of temperature and initial pH on the ethanol production (shown in Fig. 7b) clearly indicates that a proper combination of temperature and initial pH was important for production of ethanol. An increase in the temperature with initial pH increased the ethanol production gradually, but at a higher temperature the trend was reversed. The optimum for maximum ethanol production lies near the center point of the temperature and initial pH. A similar effect on the response was observed for the temperature at any level of the time. An increase in the temperature with glucose concentration up to the optimum point increased the ethanol production to maximum level and a further increase in the temperature with glucose concentration decreased the ethanol production (as shown in Fig. 7c). These three-dimensional response surface plots indicated that both of these factors were optimized nearly in the one-factor-at-α-time approach (shown in Fig. 8). Therefore, an optimum was observed near the central value of temperature, glucose concentration, and initial pH.

Optimization of fermentation conditions in *HYM*

To further validate the model accuracy and effectiveness, ethanol fermentation experiments of *Zymomonas mobilis HYM* were performed under the best conditions six times,
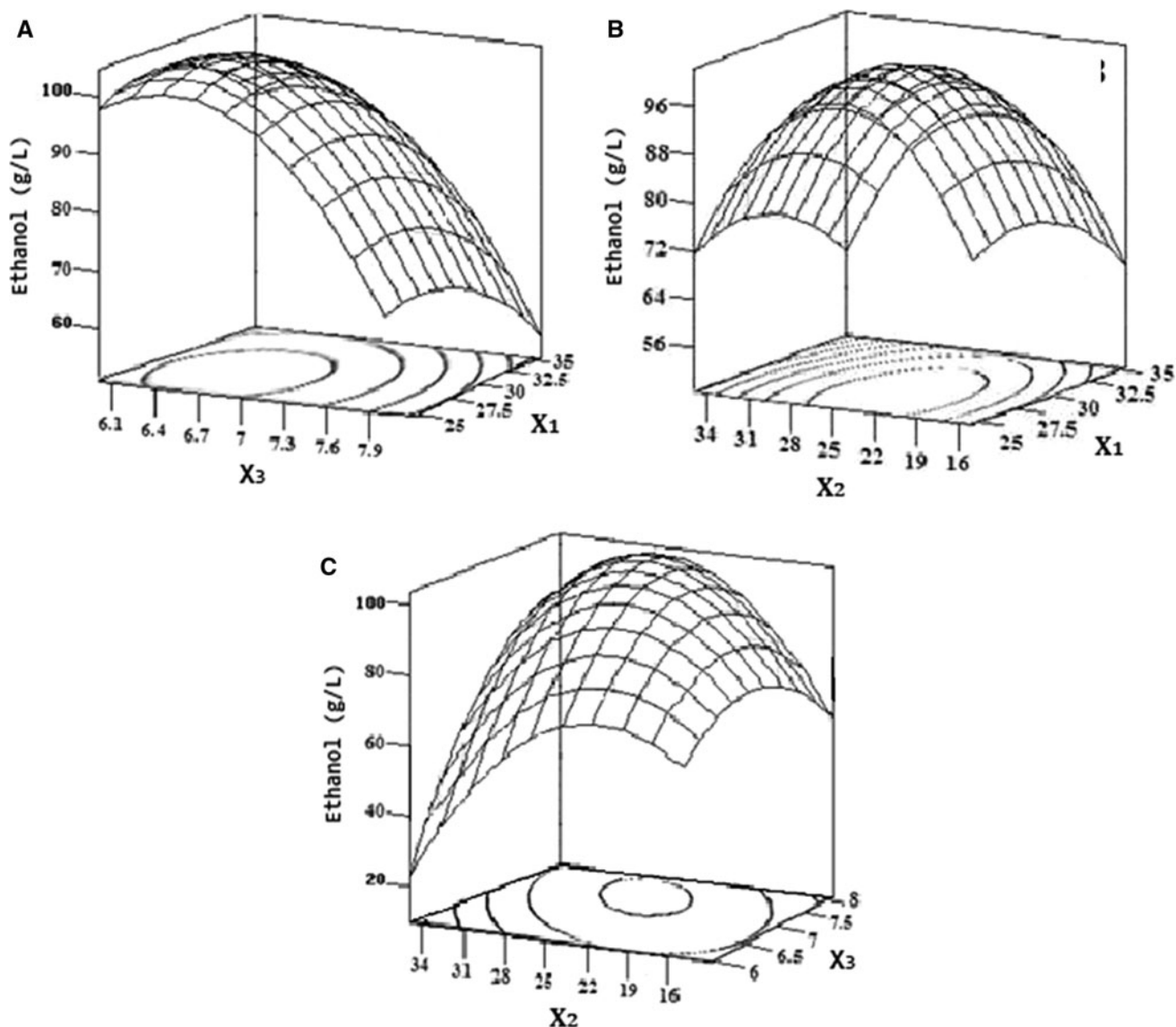
increased, the response indicated a maximum level nearly at the middle of initial pH. A further increase in the glucose concentration with initial pH resulted in a reversal of the

Fig. 8 Response surface and contour plot of the effect on the ethanol production. X_1 was temperature, X_2 was glucose concentration (g/l), X_3 was initial pH, and Y was ethanol concentration (g/l). **a** Responsive surface of $Y = f(X_1, X_3)$. **b** Responsive surface of $Y = f(X_1, X_2)$. **c** Responsive surface of $Y = f(X_2, X_3)$

with temperature, glucose concentration, and initial pH being 28.1 °C, 241 g/l, and pH 7.4, respectively. The ethanol concentrations of the six experiments were 106.5, 105.5, 107.0, 103.1, 104.5, and 103.9 g/l, respectively. These six experiments yielded an average maximum ethanol concentration of 105.1 g/l.

Discussion

Ethanol-producing bacteria Z. mobilis have attracted much attention in recent years because their growth rate is substantially higher than that of the *Saccharomyces* presently used for practical production of fuel alcohol, and with the

recent advances in biotechnology, it has the potential to play a key role in making production of ethanol much more economical [5]. In nature, Z. mobilis is found in high sugar-content solutions fermenting sugars to ethanol in a high-yield and high-productivity process. It has several advantages over yeast. Ethanol yield of Z. mobilis is as high as 97 % of theoretical yield when glucose or fructose is the substrate. It has a high specific ethanol productivity that is up to 2.5 times higher than yeast. It can tolerate up to 400 g/l glucose, up to 160 g/l ethanol, and up to 8 g/l acetic acid (at pH 6) [11]. Its ability to grow at lower pH (5.0–5.5) helps to reduce the aseptic requirements for fermentation, thus rendering the fermentation process more economical.

The growth of *Z. mobilis* has long been known to require lysine, methionine, and the whole-genome sequencing had revealed the specific reasons for these deficiencies. The only genes missing for lysine and methionine synthesis are *yfdZ* and *metB*, respectively [6]. Herein for the first time, a variant of *Z. mobilis* was achieved by genome integration of the bi-cistronic *yfdZ* and *metB* genes from *E. coli*, which lead to the fact that a strain can grow in simple chemical defined medium without the addition of amino acid. *Pfu-sHSP* is a kind of α-crystallin homologue protein that can act as a molecular chaperone, preventing membrane destabilization or aggregation of denatured proteins under heat and other stress. Therefore it can potentially be used to maintain cell viability under unfavorable conditions, such as heat shock or chemical treatments. Herein, for the first time, we proved that the *Pfu-sHSP* gene containing *Z. mobilis* showed a significantly improved tolerance to heat stress.

Although plasmids may be readily maintained in *Z. mobilis* when cultivated in mono-culture under controlled conditions, they frequently become unstable when grown in the absence of antibiotic selection pressure. Instability may be exacerbated when *Z. mobilis* has to compete with other organisms in a mixed culture. In addition, antibiotic usage for plasmid maintenance is generally perceived as undesirable for industrial application. Thus, a Tn5-based random mutagenesis system was used in this research to integrate the cloned genes into the *Z. mobilis* genome where they were maintained at a low natural copy number and were thus not over-expressed, and where, at least theoretically, they should be as stable as genome DNA.

As low-cost industry fermentations may always be carried out in rather poor, toxic, viscous, nutrient-limited mediums, successful fermentations to produce ethanol using *Z. mobilis* require not only the capability to convert sugar to ethanol with high speed and yield but also the tolerance to stresses such as high-temperature and low-nutritional conditions. These cellular characteristics are important because high temperature in summer and low nutritional concentration at the end of a batch are common in the ethanol industry. In this research, three foreign genes (*yfdZ* and *metB* from *E. coli*, *Pfu-sHSP* from *P. furiosus*) were integrated into the genome DNA of *CP4* to create the *Z. mobilis* HYM through Tn5 transposon-mediated transposition. The recombinant strain *HYM* has the following characteristics listed below at the same time: it can grow in simple chemical defined medium without the addition of amino acid; it can ferment glucose to produce ethanol with high yield; having high tolerance to high temperature and lower nutrient; the high-performance fermentation characteristics were stable up to 100 generations on nonselective medium.

The RSM with BBD was successfully used in this research to determine the importance of factors at different levels and to determine the accurate values of the fermentation conditions for the maximum ethanol production of *HYM*. The maximum ethanol concentration of *HYM* was 104.5 g/l when temperature, glucose concentration, and initial pH were 28.1 °C, 241 g/l, and pH 7.4, respectively. Compared with the predicted levels of ethanol concentration was 104.5 g/l by the BBD, the verified value was 105.1 g/l showed that the positive deviation was 0.55 %6. The good agreement between the predicted and experimental results verified the validity of the model and the existence of the optimal point. After the optimization, the ethanol production increased by 6.5 % compared to that without optimization. Theoretically, 100 g of glucose will produce 51.4 g of ethanol and 48.6 g of carbon dioxide. Under the optimized conditions, the ethanol yield of *HYM* was 0.44 g/g (ethanol/sugar) with a theoretical yield as 85.6 %, which was very close to that of the theoretical value.

Our results demonstrated that *HYM* can be an alternative cost-effective bio-ethanol producer for ethanol production that is much less sensitive to temperature and nutritional changes.

Conflict of interest The authors declare that they have no competing interests.

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