



# Compatible solutes adaptive alterations in *Arthrobacter simplex* during exposure to ethanol, and the effect of trehalose on the stress resistance and biotransformation performance

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Received: 20 July 2019 / Accepted: 10 January 2020 / Published online: 28 January 2020  
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## Abstract

Ethanol-tolerant *Arthrobacter simplex* is desirable since ethanol facilitates hydrophobic substrates dissolution on an industrial scale. Herein, alterations in compatible solutes were investigated under ethanol stress. The results showed that the amount of trehalose and glycerol increased while that of glutamate and proline decreased. The trehalose protectant role was verified and its concentration was positively related to the degree of cell tolerance. *otsA*, *otsB* and *treS*, three trehalose biosynthesis genes in *A. simplex*, also enhanced *Escherichia coli* stress tolerance, but the increased tolerance was dependent on the type and level of the stress. *A. simplex* strains accumulating trehalose showed a higher productivity in systems containing more ethanol and substrate because of better viability. The underlying mechanisms of trehalose were involved in better cell integrity, higher membrane stability, stronger reactive oxygen species scavenging capacity and higher energy level. Therefore, trehalose was a general protectant and the upregulation of its biosynthesis by genetic modification enhanced cell stress tolerance, consequently promoted productivity.

**Keywords** Trehalose · Stress tolerance · *Arthrobacter simplex* · Protection mechanism · *Escherichia coli*

## Introduction

Steroid compounds have attracted increasing attention since low-cost natural sterols (e.g., phytosterols) can be converted into valuable intermediates with improved physiological

functionality. Nonetheless, the bottleneck of steroid biotransformation remains the low solubility of the substrate. From an economic perspective, the utilization of organic solvents is a cost-effective approach to promote hydrophobic substrate dissolution on an industrial scale. Nevertheless, the amounts of organic solvents that can be added are strictly limited due to their inhibitory effects on cell growth and viability at a high dosage, which restricts the amounts of substrate that can be added, consequently resulting in unsatisfactory productivity [1]. Therefore, it is essential to find the key factors affecting strain resistance, based on which steroid transformation strains with good resistance may be further developed.

In a few model microorganisms (i.e., *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae*), modification of the level of intracellular compatible solutes, such as trehalose, glycerol, proline, glutamate, proline betaine and ectoine, has been proved to play a powerful role in maintaining homeostasis when challenged with osmotic stress. Recently, researches have provided evidence that the amounts of compatible solutes in the cell are greatly changed during exposure to organic solvents [2, 3]. However, the

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s00449-020-02286-9>) contains supplementary material, which is available to authorized users.

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changes not only strongly depend on the species of microorganism, but also they may greatly differ among different strains even though they belong to the same species [2]. In many studies, a strong connection between the amounts of compatible solutes and the levels of organic solvents resistance was observed [4–11]. Nonetheless, several conflicting results appeared. Bandara et al. [12] reported that accumulation of trehalose promoted the survival of *S. cerevisiae* at lethal ethanol concentrations but did not affect growth at sublethal concentrations. Furthermore, it was found that trehalose accumulation hardly correlated with yeast viability under long-term growth or short-term shock condition with ethanol [7, 13–15]. These investigations concluded that the relevance of compatible solutes for resistance to organic solvents was rather complex, and was closely dependent on the selected strain as well as the type and amount of organic solvents.

Considerable progress has been made to enhance organic solvents tolerance in a variety of microorganisms by genetically engineering the compatible solute metabolic pathways or by exogenous addition of the compatible solutes. For example, the accumulation of trehalose or proline by gene manipulation efficiently elevated ethanol tolerance in different yeast [16–20] and *E. coli* cells [21]. Supplementation with a mixture of compatible solutes or single compounds (such as glycine, betaine and proline) remarkably enhanced ethanol tolerance in *S. cerevisiae* [22] and *E. coli* [21, 23, 24]. Nevertheless, relevant studies with Gram-positive bacteria, especially industrially used strains have been practically absent so far.

*Arthrobacter simplex* is known as the most exploited and primary microbe for the steroid  $\Delta^1$ -dehydrogenation process, by which reaction the anti-inflammatory activity of the steroid is greatly enhanced. During *A. simplex* industrial application, ethanol is mostly added as a solvent to dissolve the hydrophobic substrate (e.g., cortisone acetate). During the biotransformation, the concentration of ethanol is usually no more than 8% (vol/vol) and the incubation time ranges from 24 to 60 h, depending on the amount of the substrate to be converted. In our previous work, the levels of trehalose and glycerol were elevated when *A. simplex* cells were grown under moderate ethanol stress [1, 25]. Nevertheless, the changes in compatible solutes profile (not merely trehalose and glycerol) under various ethanol stresses (such as shock by high concentration) were not fully discussed. Moreover, the present data are insufficient to conclude that the accumulation of compatible solutes is the critical determinant of ethanol resistance in *A. simplex*. In this paper, the adaptive responses of four kinds of compatible solutes were systematically investigated in *A. simplex* CPCC 140,451 during exposure to various ethanol stresses, including long-term growth at mild concentration and short-term shock by a high concentration of ethanol. Among four kinds of compatible

solutes, the influence of trehalose on cell stress tolerance and strain biotransformation performance was investigated. The mechanisms of the protection by trehalose were further revealed. Our study, for the first time, comprehensively described the adaptive modifications of multiple compatible solutes and established the protection role of trehalose in *A. simplex* at various ethanol conditions. Our results showed that the genetic modification of trehalose biosynthesis is a general and efficient strategy for enhancing stress tolerance in different kinds of bacteria (both Gram-negative and Gram-positive bacteria).

## Materials and methods

### Strains, plasmids, media and culture conditions

Strains and plasmids used in this study are shown in Table S1. Genomic DNA was extracted from *A. simplex* CPCC 140,451 using TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China), which was employed as templates to amplify the selected genes (*otsA*, *otsB* and *treS*) using the corresponding primers shown in Table S2. The PCR products were purified with a Cycle-Pure Kit (Omega Bio-Tek, Norcross, USA), extracted and cleaved by *Bam*H I/*Xba* I (TaKaRa Biotech, Beijing, China). The shuttle vector pART2 [26] is used to construct the recombinant plasmids and the construction workflow is presented in Fig. S1A. The resulting plasmids were confirmed by PCR, double enzyme digestion (Fig. S1B) and DNA sequencing. Then, the verified plasmids were transformed into *A. simplex* by electroporation as described in our previous work [1], or into *E. coli* BL21 by heat shock transformation. The strain only harboring the empty plasmid pART2 was used as the control strain.

The wild-type *A. simplex* strain was cultured in the same medium as described in our previous work [27], while all engineering strains were grown in LB medium containing kanamycin (50  $\mu$ g/mL). For *A. simplex* and *E. coli* strains, cells were cultured at 32 °C/160 rpm and at 37 °C/200 rpm, respectively.

### *A. simplex* wild-type strain growth and survival ability under ethanol stress

Cells were grown in a 250 mL Erlenmeyer flask containing 50 mL of medium until mid–late exponential phase and the cultures were transferred into 250 mL Erlenmeyer flask containing 50 mL of medium using 5% (vol/vol) inoculum size. When the cell growth entered into the mid–late exponential phase (approximately 18 h,  $OD_{600} = 3.2$ ), ethanol was added to a final concentration of 4%, 6%, 8%, 10% (vol/vol), respectively, and the cells were continued to be cultivated. During the whole process,  $OD_{600}$  values were

measured. The cell survival after 15% (vol/vol) ethanol shock for 0.5 h or 1.0 h was determined by a CFU method as described in our previous work [1].

### Measurement of intracellular compatible solutes

The cells were grown in the absence or presence of ethanol with different concentrations. The cultures were harvested at the indicated times and washed with phosphate buffer saline (PBS, pH 7.2) twice. The compatible solutes were analyzed according to commonly used methods [28].

### Quantitative real-time PCR assay

The cells were cultivated under specific conditions and the cultures were harvested at the indicated times. After centrifugation, the resulting cells were resuspended in phosphate buffer saline (PBS, pH 7.2). 2 ml of cell suspension was removed, rapidly chilled on ice, and washed with ice-cold distilled water. The pellets were immediately frozen in liquid nitrogen and kept at  $-80\text{ }^{\circ}\text{C}$  until preparation of total RNA. The RNA was extracted from the samples using RNAiso plus (TaKaRa Biotech, Beijing, China) and then reverse-transcribed using the ProtoScript First Strand cDNA Synthesis Kit (TaKaRa Biotech, Beijing, China) according to the protocol recommended by the supplier. For real-time quantitative polymerase chain reaction (PCR), the synthesized cDNA was amplified with oligonucleotide primers for each gene using a BIO-RAD Real-Time PCR System (Applied Biosystems, Foster City, USA). The nucleotide sequences of primers for the selected and reference genes were designed using DNAMAN software and listed in Table S3. The quantitative real-time RT-PCR (qRT-PCR) was conducted on a Step-One TM Real-Time PCR System (Applied Biosystems, New York, USA) using One-Step SYBR Prime-Script RT-PCR Kit (TaKaRa Biotech, Beijing, China). The following amplification conditions were employed: an initial incubation at  $95\text{ }^{\circ}\text{C}$  for 30 s; 40 cycles of PCR at  $95\text{ }^{\circ}\text{C}$  for 10 s,  $60\text{ }^{\circ}\text{C}$  for 30 s; and a final incubation at  $95\text{ }^{\circ}\text{C}$  for 15 s,  $60\text{ }^{\circ}\text{C}$  for 60 s. PCR amplification was detected by SYBR Green fluorescence dye (Takara Bio Dalian CO, Ltd, China). The 16S rRNA gene was used as the housekeeping gene (internal control) to normalize for differences in total RNA quantity. Relative gene expression levels were calculated by the comparative Ct method ( $2^{-\Delta\Delta C_t}$  method) [29] and then normalized either to the levels of corresponding genes in the non-ethanol-treated cells or the levels of corresponding genes in the control strain. PCR reactions were carried out in triplicate with each of three different cDNA samples.

### The cell growth assay in the absence of ethanol

The overnight cultures of the trehalose-overproducing strains and the control strain were transferred into 50 mL of LB medium containing kanamycin ( $50\text{ }\mu\text{g/mL}$ ) in a 250-mL Erlenmeyer flask to a starting  $\text{OD}_{600}$  of 0.02. Then, the cells were cultivated under non-ethanol condition and  $\text{OD}_{600}$  values were monitored during growth.

### Determination of cell tolerance to various abiotic stresses

Cell growth and survival ability under various abiotic stress conditions were used as indicators of stress tolerance, which were determined as previously [1]. For cell growth analysis, the overnight cultures were diluted to a final  $\text{OD}_{600}$  of 0.02 in LB medium supplemented with kanamycin ( $50\text{ }\mu\text{g/mL}$ ) and various challenging substances, and then grown at  $32\text{ }^{\circ}\text{C}$ . For *A. simplex* strains, the challenging substance included 6% (vol/vol) ethanol or methanol, 0.6 M NaCl and 0.025% (vol/vol)  $\text{H}_2\text{O}_2$ . For *E. coli* strains, the challenging substance included 4% (vol/vol) ethanol, 5% (vol/vol) methanol, 0.6 M NaCl, 0.025% (vol/vol)  $\text{H}_2\text{O}_2$ , 1 M sorbitol and  $\text{pH}=5.0$ . During the process,  $\text{OD}_{600}$  was monitored to obtain cell growth curves. In the shocking experiment, *A. simplex* cells were challenged with 20% ethanol, 15% methanol, 2.0 M NaCl or 0.1% (vol/vol)  $\text{H}_2\text{O}_2$  for 1.0 h, respectively, while *E. coli* strains were subjected to 10% (vol/vol) ethanol, 15% (vol/vol) methanol, 2.5 M NaCl, 0.1% (vol/vol)  $\text{H}_2\text{O}_2$ , 3 M sorbitol and  $\text{pH}=3.0$  for 30 min, respectively. The cell survival was determined by a CFU method [1].

### Bioconversion of cortisone acetate (CA) to prednisone acetate (PA) by *A. simplex*

The resting cells were prepared in three consecutive steps for bioconversion of cortisone acetate (CA) to prednisone acetate (PA): (1) inoculation of strains in 50 mL of LB medium in a 250-mL Erlenmeyer flask and cultivation at  $32\text{ }^{\circ}\text{C}$  and 160 rpm for 36–40 h; (2) 1 mL of the cultures from step 1 was used to inoculate 50 mL of LB medium in a 250 mL Erlenmeyer flask. The cells were then cultivated until the  $\text{OD}_{600}$  of the culture reached 1.2–1.5, and then 0.05% (w/v) CA used as an inducer was added. And finally, (3) after incubation for 16 h at  $32\text{ }^{\circ}\text{C}$  and 160 rpm, the cells were harvested, washed, and resuspended in PBS buffer to bring the  $\text{OD}_{600}$  to 2.0. Next, 30 mL of the cell suspension was added into a 100-mL Erlenmeyer flask and pre-incubated at  $32\text{ }^{\circ}\text{C}$  and 160 rpm for 15 min to maintain temperature equilibrium. Then, four systems were employed, including (1) system I: 2 g/L of CA dry powder; (2) system II: 2 g/L of CA dissolved in 4% (vol/vol) ethanol; (3) system III: 6 g/L of CA dissolved in 8% (vol/vol) ethanol; (4) system IV: 8 g/L of CA

solved in 10% (vol/vol) ethanol. The bioconversion was performed at 32 °C/180 rpm and the samples were withdrawn at the indicated time. The substrate and product amounts were analyzed by high-performance liquid chromatography (HPLC) and the initial PA production rate was calculated from the slope of initial linear fragments of accumulation time curves according to our previous method [1].

On the other hand, the growing cells were prepared according to the above step 1 and 2 for the resting cells. When the  $OD_{600}$  of the cultures reached 2.0, CA and ethanol were added. For growing cells, system V (35 g/L CA dissolved in 10% (vol/vol) ethanol) was used and the other bioconversion conditions were maintained.

### Viable cell count and dehydrogenase activity analysis of *A. simplex* strains

The resting cells ( $OD_{600}$  at approximately 2.0) were, respectively, added to systems I, II, III, and IV, all without substrate. After transformation at 32 °C and 180 rpm for 12 h or 30 h, the cultures were withdrawn to determine the number of viable cells using a CFU assay. Meanwhile, the dehydrogenase activity was tested by the triphenyl tetrazolium chloride (TTC) method. The procedure was carried out as follows. The cell cultures were harvested at the indicated condition. Then, 1 mL was pipetted from each sample into four test tubes (tests were carried out in triplicates with a control with each sample). PBS buffer (1 mL) was added to all test tubes. Then, 2 mL of TTC–glucose solution was added to the sample tubes (1 mL of distilled water was added to the control tube). The test tubes were gently swirled to mix the content and incubated at 37 °C for 30 min. The reaction was terminated by 0.5 mL formaldehyde. Samples were then centrifuged for 10 min to separate the cells from the liquid medium and the supernatant was discarded. TF extraction was carried out three times using 80% acetone each time. All samples were vortexed to aid in the extraction of TF from the cells. Samples were centrifuged again and the supernatants from the extraction were combined. The absorbance of the combined supernatants was measured at 485 nm using a spectrophotometer. The dehydrogenase activity of the different samples was expressed as the ratio of  $OD_{485}$  of the treated sample to the control. The relative dehydrogenase activity was expressed as the ratio of the value in the presence of ethanol divided by the value in the presence of PBS buffer at the same time [1].

### Cell ultrastructure observation and membrane fluidity determination

The cell ultrastructure was directly observed using a JSPM-5200 AFM (Bihe International Trading Limited, Shanghai, China). Cell fluidity was assessed by measuring

the fluorescence polarization of the cells labeled with the hydrophobic molecular probe DPH (1,6-diphenyl-1,3,5-hexatriene). Details about the procedure are shown in our previous work [25].

### The measurement of ROS content, antioxidant enzyme activity and ATP level

The cells were disrupted using a Scientz-IID sonicator (Ningbo Scientz Biotechnology CO, Ltd, Ningbo, China) on an ice bath for 15 min (5 s each with a 5 s interval between each treatment) at 300 W. Unbroken cells and cell debris were removed by centrifugation at 5000×g for 20 min at 4 °C. The resulting supernatants were immediately employed to determine ROS content, and the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) as described in our previous work [1].

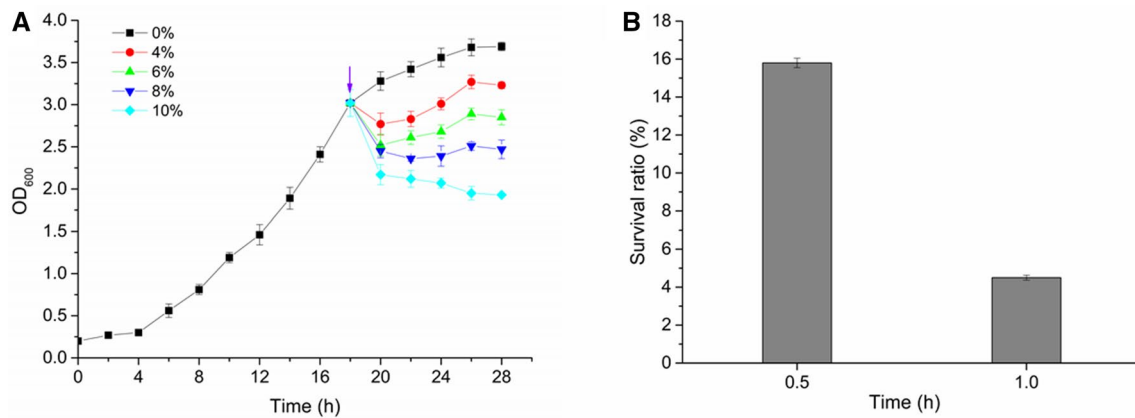
### Statistical analyses

All experiments were repeated at least three times in independent experiments. In each experiment, assays were performed in at least three replications. The statistical analysis was performed by analysis of variance (ANOVA) and the comparison of the mean values was accomplished by the Duncan test ( $P < 0.05$ ).

## Results and discussion

### Growth and survival of *A. simplex* under ethanol stress

As shown in Fig. 1a, the exponential phase of cell growth without ethanol began at 4 h, and the highest  $OD_{600}$  of the cultures (3.68) was obtained at 26 h. At  $\leq 8\%$  (vol/vol) ethanol, the  $OD_{600}$  of cultures sharply decreased within the first 2 h (18–20 h) and then gradually increased with the prolonged time. The peak value, which was detected at 26 h, was negatively correlated with ethanol concentration. Moreover, the peak value of ethanol-treated cells was lower than that of untreated cells. These data indicated that ethanol indeed affected cell growth, but the cells still could retain a little viability within the tested concentration range of ethanol. When ethanol concentration was up to 10% (vol/vol), the  $OD_{600}$  of cultures decreased during the whole process, implying that cell growth was severely suppressed. The survival ratio of cells after 15% (vol/vol) ethanol shock for 0.5 and 1.0 h rapidly decreased to 15.8% and 4.5%, respectively (Fig. 1b). These data suggested that the inhibition role of ethanol on *A. simplex* was concentration- and time-dependent, which helped us to select the appropriate ethanol stress conditions for the following analysis.



**Fig. 1** a *A. simplex* growth curves when different concentrations of ethanol were added at mid-late exponential phase (18 h). The arrow indicates the time of ethanol addition; (b) *A. simplex* survival ratio

after shock by 15% (vol/vol) ethanol for 0.5 or 1.0 h, respectively. All data represent the mean values from three independent experiments

### Adaptive modifications of compatible solutes in *A. simplex* under ethanol stress

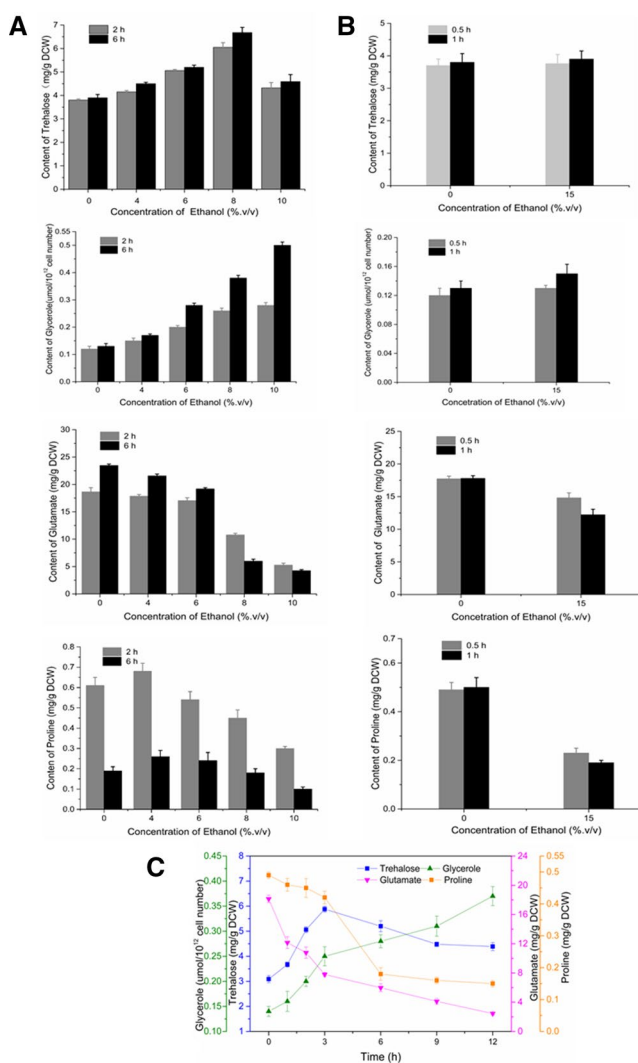
Four kinds of compatible solutes, including trehalose, glycerol, proline, and glutamate, were simultaneously detected, and each compound exhibited a specific change under various stressful ethanol conditions. Figure 2a shows that in cells grown at mild ethanol concentrations, the level of trehalose rapidly increased and peaked [6.68 mg/g dry cell weight (DCW)] at 8% (vol/vol) ethanol for 6 h, which was 71% than that in the untreated cells. At 10% (vol/vol) ethanol, trehalose content rapidly decreased but was still higher than that in non-stressed cells. Glycerol accumulation was prominently concentration- and time-dependent. The maximum value ( $0.50 \mu\text{mol}/10^{12}$  cell number), which was 2.8-fold higher than that of untreated cells, was achieved at 10% (vol/vol) ethanol for 6 h. The glutamate amount reduced gradually with increasing ethanol concentration but remained at a high level (17.05–23.46 mg/g DCW) at 6% (vol/vol) of ethanol. A sharp decline in glutamate content was then observed, and the lowest value (4.24 mg/g DCW), which was only 18.1% of that under non-stressed condition, was reached at 10% (vol/vol) ethanol for 6 h. Proline content slightly increased at 4% (vol/vol) of ethanol and then gradually dropped with elevated ethanol concentration and prolonged time. The minimum (0.10 mg/g DCW), which was only 52.6% of that under non-stressed condition, was obtained at 10% (vol/vol) ethanol for 6 h. As shown in Fig. 2b, after 15% (vol/vol) of ethanol shock, trehalose only slightly increased, whereas the change patterns in the other three compatible solutes were very similar to that during growth at mild concentration.

The change process in each compatible solute was further analyzed. Ethanol [6% (v/v)] was added at the mid-late exponential cell growth phase because the cell viability of *A. simplex* was maintained at a certain level at this ethanol

concentration, and the compatible solute contents were also considerably modified. After ethanol addition, trehalose rapidly accumulated and peaked at 3 h (5.88 mg/g DCW) and gradually decreased with time but was still higher than that in untreated cells. Glycerol content increased remarkably and peaked at 12 h ( $0.37 \mu\text{mol}/10^{12}$  cell number), which was 1.6-fold higher than that in untreated cells. The glutamate and proline levels were consistently reduced during the whole process (Fig. 2c).

The expression levels of the related genes were examined by qRT-PCR to determine whether the accumulation of trehalose and glycerol resulted from the metabolism of *A. simplex*. In *A. simplex*, the two biosynthetic pathways of trehalose, which included OtsA–OtsB and TreS pathways, were first identified. In the OtsA–OtsB pathway, an  $\text{Mg}^{2+}$ -dependent trehalose-6-phosphate synthase, the product of the *otsA* gene EC 2.4.1.15, initially catalyzed the enzymatic condensation of the precursor glucose-6-phosphate and UDP-glucose to yield trehalose-6-phosphate. Subsequent dephosphorylation by trehalose-6-phosphate phosphatase, the product of the *otsB* gene EC 3.1.3.12, led to the formation of free trehalose. In the TreS pathway, the  $\alpha$  (1 → 4) link in maltose was converted to an  $\alpha$  (1 → 1) link to form trehalose by trehalose synthase, the product of the *treS* gene EC 5.4.99.16. Glycerol was phosphorylated to glycerol-3-phosphate (G-3-P) by glycerol kinase (the product of the *glpK* gene, EC 2.7.1.30) or glycerophosphoryl diester phosphodiesterase (the product of the *glpQ* gene, EC 3.1.4.46), respectively, G-3-P was then converted to dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase (the product of the *glpA* gene, EC 1.1.5.3) or 1-acyl-sn-glycerol-3-phosphate acyltransferase (the product of the *gpsA* gene, EC 2.3.1.51, respectively. As shown in Fig. S2, the transcriptional levels of trehalose biosynthesis genes were all upregulated when cells were grown at 6%

**Fig. 2** The intracellular compatible solutes contents of *A. simplex* after addition of different concentrations of ethanol at mid-exponential phase for 2 or 6 h (a) or after shock by 15% (vol/vol) ethanol for 0.5 or 1.0 h (b), respectively; (c) The change curves of intracellular compatible solutes contents after addition of 6% (vol/vol) of ethanol at mid-exponential phase. All data represent the mean values from three independent experiments



(vol/vol) of ethanol for 3 h. The highest relative expression level was observed in *otsB* (13.23), followed by *otsA* and *treS* (approximately 5.60). The transcriptional levels of glycerol degradation genes were all downregulated. The lowest relative expression level was detected in *gpsA* (0.22), followed by *glpQ* (0.68) and *glpA* (0.80). Only a slight drop was observed in *glpK* (0.95). These results were consistent with the metabolite examination results, suggesting that the accumulation of trehalose and glycerol was ethanol-induced and regulated at the level of biosynthesis rather than uptake.

In most *S. cerevisiae* strains, ethanol generated a variable effect on the expression levels of genes involved in trehalose or glycerol metabolism, consequently accumulating more protective substances [3, 6, 7, 30, 31]. Consistent with our data, the trehalose levels in *S. cerevisiae* strains were elevated within the first 2 days when grown at 9% (vol/vol) ethanol and then decreased with time, whereas the proline level continuously decreased with

time [19]. However, the trehalose content in *S. cerevisiae* was enhanced with the addition of 9% ethanol at the exponential growth phase, whereas the levels of glycerol and glutamate decreased within the tested time (6 h). This result was further proved by the expression level analysis of genes involved in trehalose and glycerol metabolism. Proline was accumulated after ethanol addition. Cells did not produce proline because the expression levels of biosynthesis-related genes did not obviously change, whereas those of genes participated in transport systems were drastically upregulated, which supported the fact that proline accumulation was primarily due to an increase in its uptake from the nutrient medium [28]. The decrease in trehalose content had also been observed in yeast strains exposed to ethanol [8, 15]. In *Saccharomycopsis fibuligera*, neither the activation of gene expression nor the change in trehalose content was observed after ethanol addition [31].

### Effects of trehalose on stress tolerance in *A. simplex* and *E. coli*

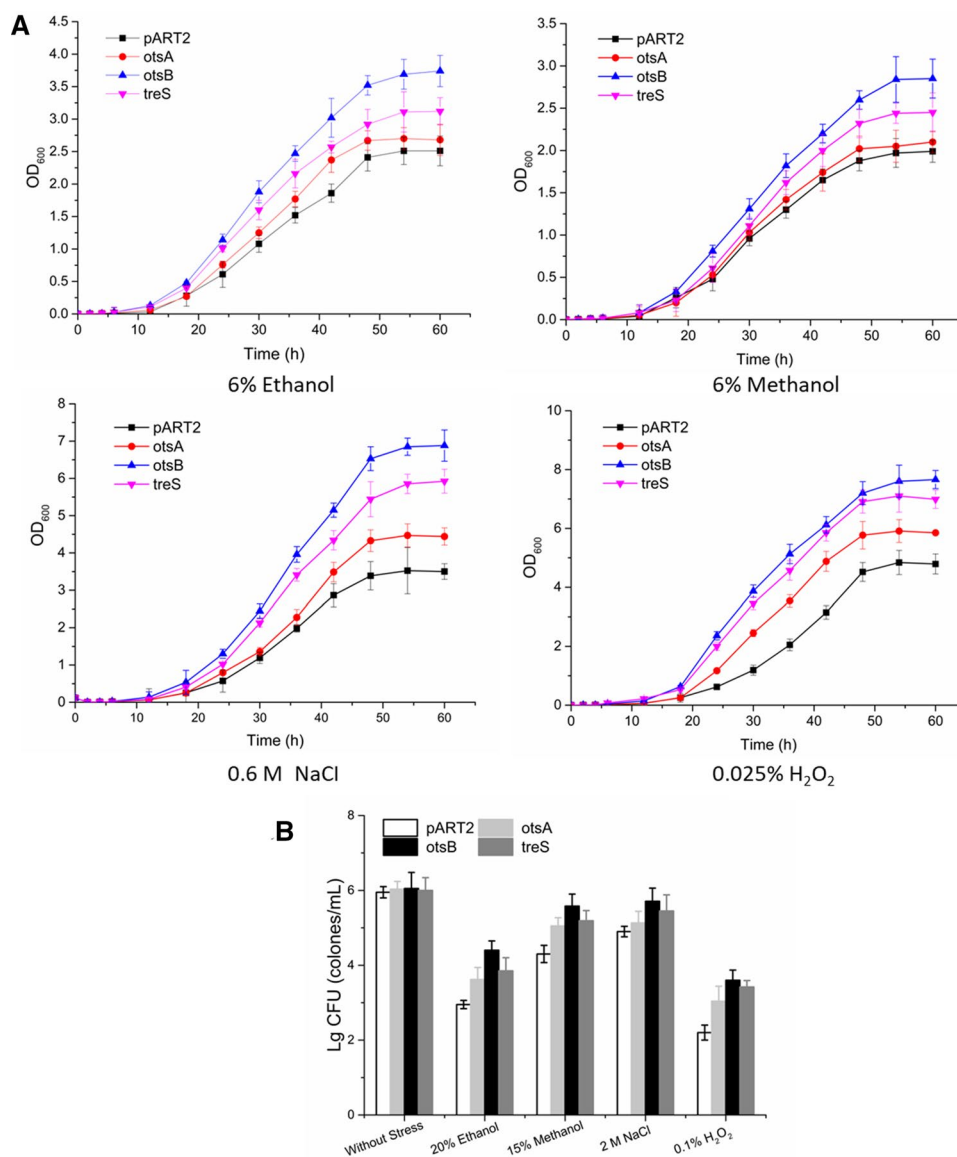
By individually overexpressing *otsA*, *otsB*, and *treS* genes, three *A. simplex* strains accumulating trehalose were constructed. As shown in Fig. S3A, their growth profiles did not differ remarkably from that of the control strain. A similar phenomenon has also been observed in *S. cerevisiae*, where a series of mutants with different trehalose contents exhibited almost identical growth behavior without ethanol [4]. Conversely, Kim et al. [16] reported that the *S. cerevisiae* strain with more trehalose content possessed better growth than the wild-type strain.

For all *A. simplex* strains, the cells at mid-exponential phase (30 h) produced the highest trehalose, followed by the cells at the stationary (50 h) and early exponential phases

(20 h; Fig. S3B). However, the capacity of yeasts to produce trehalose had been observed at the stationary [11, 14, 31] and the mid-exponential [32] phases. At all tested time points, the *otsB*-overexpressing strain produced the highest trehalose (5.6–6.8 mg/g DCW), followed by *treS*- (5.2–6.1 mg/g DCW) and *otsA*-overexpressing strain (4.7–5.6 mg/g DCW), respectively, which were much higher than that of the control strain (3.8–4.0 mg/g DCW) (Fig. S3B). Consistent with the metabolite content result, the most upregulated gene expression was observed in *otsB* in the corresponding over-expression strain, which was followed by *treS* and *otsA* at approximately 120- and 100-fold (Fig. S4). As expected, the overexpression of the selected genes did not change the expression levels of the two other genes (data not shown).

As shown in Fig. 3, three gene-overexpressing *A. simplex* strains (*otsA*, *otsB*, and *treS*) achieved better growth

**Fig. 3** **a** The cell growth curves of *A. simplex* strains in LB medium supplemented with different challenging substances, including 6% (vol/vol) ethanol or methanol, 0.6 M NaCl and 0.025% (vol/vol) H<sub>2</sub>O<sub>2</sub>; **(b)** The colony number of *A. simplex* strains after shock by various abiotic stresses for 1 h. All data represent the mean values from three independent experiments



at 6% (vol/vol) of ethanol or methanol or higher survival after shock by 20% (vol/vol) of ethanol or 15% (vol/vol) of methanol for 1 h compared with the control strain. The elevated trehalose levels also conferred *A. simplex* cells with improved resistance to oxidative and salt stresses. A positive correlation was found between the trehalose contents and the stress tolerance level in *A. simplex*. Conversely, the intracellular trehalose levels are irrelevant to ethanol tolerance in *S. pombe* [14], *S. cerevisiae* [13], sake yeast [7], and tropical yeast [15].

Three trehalose biosynthesis genes from *A. simplex* were individually introduced into *E. coli*, a Gram-negative bacterium and a model microorganism. By RT-qPCR analysis, the expression levels of *otsA*, *otsB*, and *treS* in the corresponding *E. coli* engineered strains harvested at the late-log phase (about 16 h) were upregulated by 13.2-, 18.7-, and 16.4-fold, respectively, compared with the control strain. Results indicated that these genes were successfully expressed in *E. coli*. As shown in Fig. S5, similar growth characteristics were observed in all strains. As shown in Fig. 4, the heterologous expression of three trehalose biosynthesis genes from *A. simplex* exerted remarkably diversified effects on the long-term growth of *E. coli* at mild ethanol concentration, but the change patterns did not follow a defined tendency. Similar results were observed after short-term shock by high ethanol concentration. The gene effects on cell tolerance were distinctly different between *A. simplex* and *E. coli*. For instance, the introduction of three genes could remarkably increase *E. coli* tolerance to salt and osmotic stresses, but a slight variation occurred in the tolerance to oxidative and acid stresses. The expression of *otsA* hardly affected cell growth at 4% (vol/vol) of ethanol but greatly increased cell survival after shock by 10% (vol/vol) of ethanol, whereas the expression of *otsA* could increase cell growth at 5% (vol/vol) of methanol but decrease cell survival after shock by 15% (vol/vol) of methanol. *otsB* showed the best effects on cell growth at 4% ethanol, but *otsA* exerted the most influence on cell growth at 5% methanol. In summary, the horizontal effects of three trehalose biosynthesis genes presented evident strain and stress dependence.

In *S. cerevisiae* and *S. pombe*, trehalose overproduction by manipulating genes that participated in biosynthesis, degradation, or transport has effectively enhanced ethanol tolerance [4, 5, 9, 16–18, 20]. *E. coli* and *Pseudomonas putida* dried with trehalose were shown to be tolerant to organic solvents [33, 34]. This finding proves that trehalose may have a common protective role in response to multiple stress stimuli. In sake yeast, strains with more trehalose have shown higher resistance to salt and oxidative stresses in addition to ethanol [7]. However, the accumulated trehalose in *S. cerevisiae* has contributed to strong tolerance to ethanol and salt [9] but not to oxidative stress [4]. The overexpression of *otsA* or *otsB* or both genes has previously been shown

to accumulate additional trehalose and improve *E. coli* tolerance to ethanol [21]; heat and osmotic stresses, such as salt and sugar [35]; desiccation [36]; and crude glycerol [37]. This study first demonstrated the effects of these two genes from *A. simplex* on *E. coli* stress tolerance. The role of the *treS* gene in *A. simplex* on the stress tolerance of *A. simplex* and *E. coli* was reported for the first time.

### Effects of trehalose on $\Delta^1$ -dehydrogenation performance

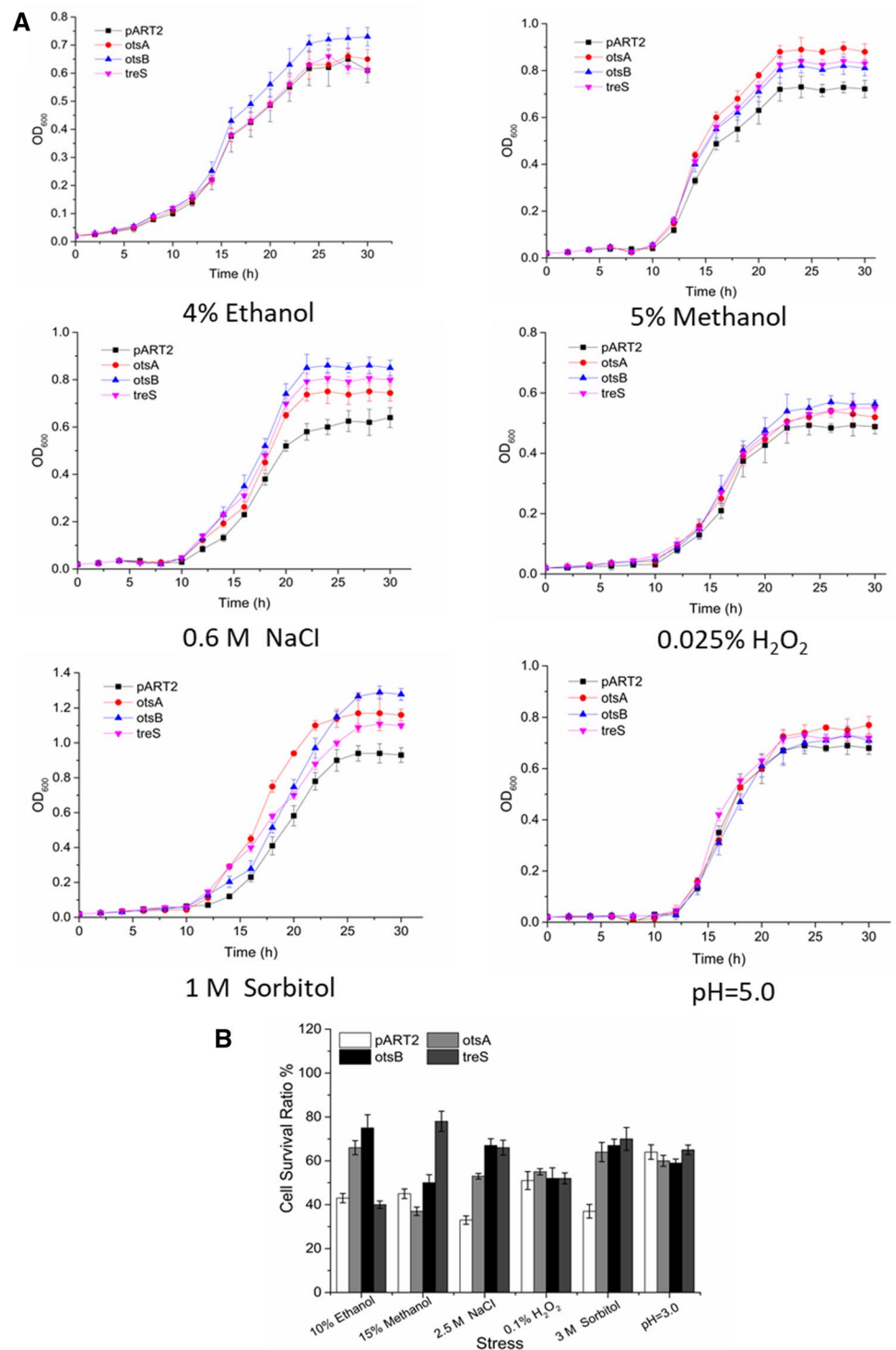
The  $\Delta^1$ -dehydrogenation reaction from CA to PA was selected as a model to evaluate the biotransformation performance of *A. simplex*. The resting cells were employed as biocatalysts to minimize the difference in the physiological status among different strains. As shown in Fig. 5, the biotransformation processes of all strains from CA to PA were very similar in systems I and II, but the strains in system II displayed higher initial production rate than that in system I. It could be explained by an increase in the dissolution rate of hydrophobic substrate using ethanol as a cosolvent. In system III, the overexpressing strains exhibited superior biotransformation performance relative to the control strain, among which the *otsB* strain presented the highest initial production rate ( $0.462 \text{ g L}^{-1} \text{ h}^{-1}$ ) and the maximal PA production ( $4.05 \text{ g L}^{-1}$ ) at 48 h, followed by *treS* and *otsA* strains. At system IV, *otsB* and *treS* strains achieved their corresponding peak of 5.10 and  $3.51 \text{ g L}^{-1}$ , respectively at 36 h, whereas *otsA* and the control strains almost lost their biotransformation ability. The biotransformation productivity of the *otsB* strain was further evaluated in practical PA production, where the growing cells were used as biocatalysts, and system V [35 g  $\text{L}^{-1}$  CA dissolved in 10% (vol/vol) ethanol] was employed. After 48 h, the maximum PA by *otsB* strain achieved  $12.85 \text{ g L}^{-1}$ , whereas only a trace amount of PA was detected in the control strain within the entire process. The engineered industrial *S. cerevisiae* strains with more intercellular trehalose achieved greatly higher fermentation rates and ethanol yields than their wild-type strain during very high gravity fermentation [20]. The overexpression of *otsBA* has endowed *E. coli* cells with the capacity to tolerate the toxicity of crude glycerol and consequently led to higher  $\beta$ -carotene production [37].

### Effects of trehalose on the cell viability of *A. simplex* strains in biotransformation systems

The viable cell counts of the control and *otsB* strains during exposure to various biotransformation systems were tested to verify whether the superior biotransformation performance was due to the enhanced tolerance to complex stresses during the biotransformation process. As shown in Fig. S6A, after 12 h in system II, the viable cell count of *otsB* strain



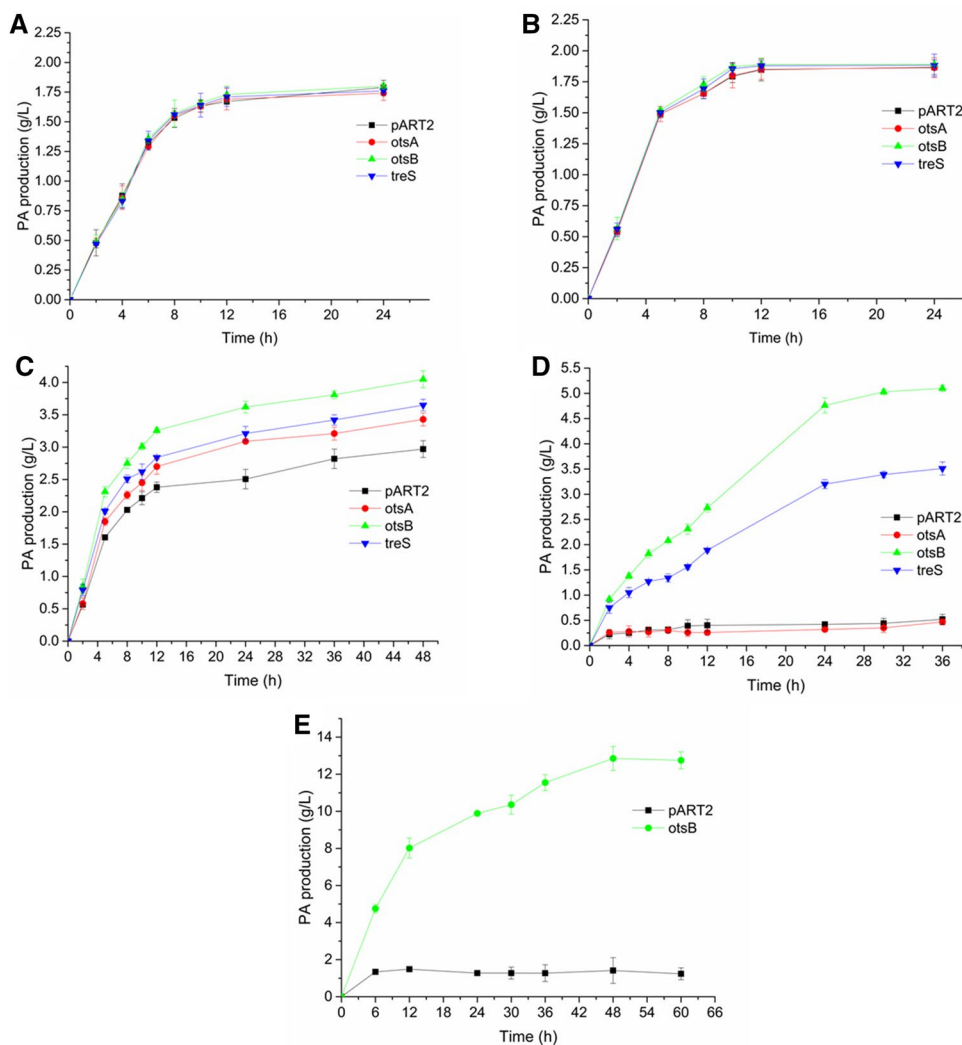
**Fig. 4 a** The cell growth curves of *E. coli* strains in LB medium supplemented with different challenging substances, including 4% (vol/vol) ethanol, 5% (vol/vol) methanol, 0.6 M NaCl, 0.025% (vol/vol) H<sub>2</sub>O<sub>2</sub>, 1 M sorbitol and pH=5.0; **(b)** The colony number of *E. coli* strains after shock by various abiotic stresses for 0.5 h. All data represent the mean values from three independent experiments



was very similar to that of the control strain, which was slightly decreased but still retained at the same magnitude relative to PBS treatment. Results suggested that the complex stresses in system II were too low to induce evident toxicity to cells. In systems III, the viable cell count of the otsB strain considerably decreased by more than two magnitudes, but conspicuous reduction by more than three magnitudes

occurred in the control strain, implying that the complex stresses in system III severely inhibited cell viability. Less viable cell counts of both strains were detected in system IV, where the viable cell count of otsB strain was more than 10<sup>2</sup> CFU mL<sup>-1</sup> even after 30 h, whereas the value of the control strain was sharply decreased to below 10 CFU mL<sup>-1</sup>. These observations coincided well with PA production by

**Fig. 5** The PA production curves of *A. simplex* strains in different biotransformation systems using the resting cells (a–d) or growing cells (e) as the biocatalysts. **a** System I where 2 g/L of CA dry powder without any cosolvent was added; **b** system II where 2 g/L of CA dissolved in 4% (vol/vol) ethanol was added; **c** system III where 6 g/L of CA dissolved in 8% (vol/vol) ethanol was added; **d** system IV where 8 g/L of CA dissolved in 10% (vol/vol) ethanol was added; **e** system V where 35 g/L dissolved in 10% (vol/vol) ethanol was added. All data represent the mean values from three independent experiments



both strains in different systems (Fig. 5). In addition, the viable cell count of both strains after exposure to systems without CA was examined, where the biocatalysts challenged with only a single ethanol stress. The viable cell counts of both strains in the systems without CA changed in a similar manner with those in corresponding systems with CA. More than 4% ethanol exerted substantial toxicity to cells, but the otsB strain always exhibited higher ethanol resistance than the control strain within the test range of ethanol concentration. In comparison with the viable cell counts of each strain subjected to a single ethanol stress, the values of each strain exposed to complex stresses slightly decreased but remained at the same order of magnitude (Fig. S6A). This result implied that > 4% (vol/vol) ethanol, instead of the substrate (CA) or product (PA), was a major stress factor during biotransformation process, which was consistent with our previous work [1].

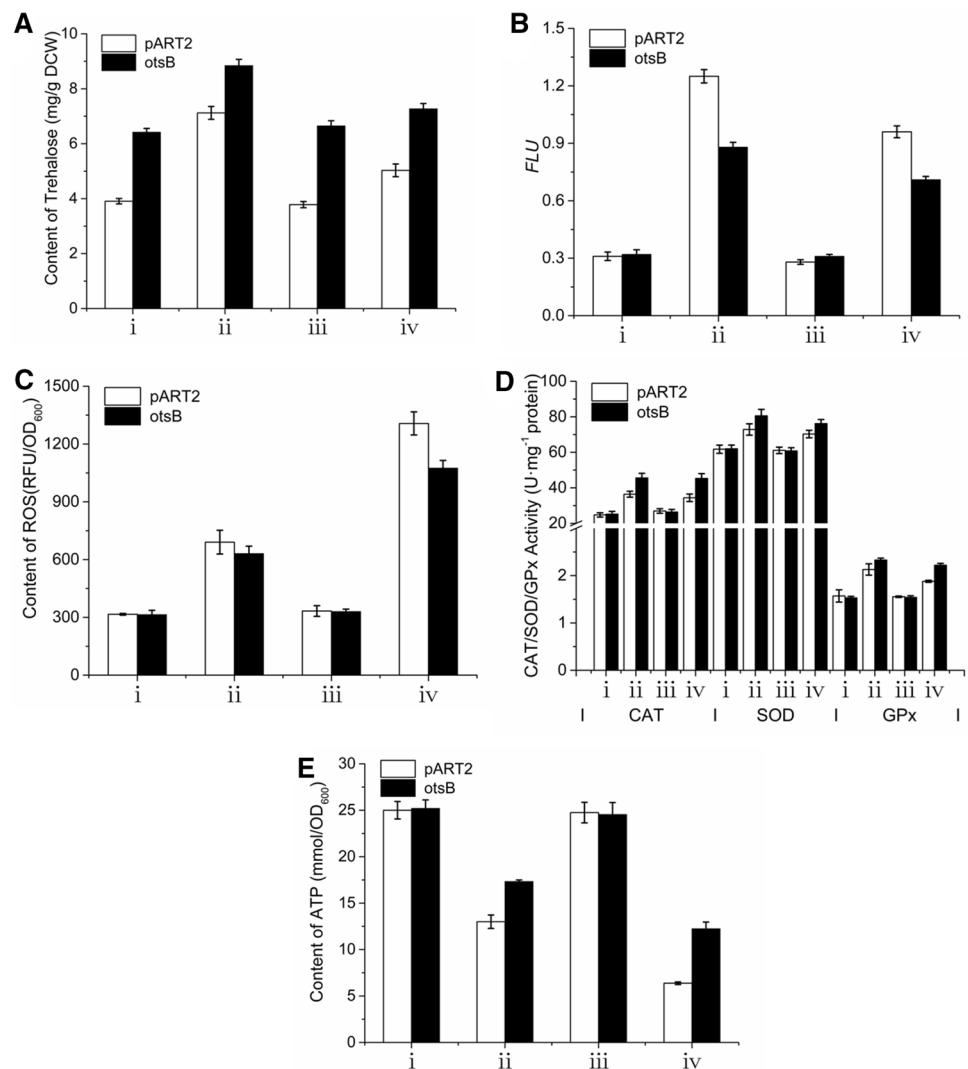
As an index reflecting basic metabolic ability, the activity of dehydrogenase was further assayed at different ethanol concentrations. The inhibitory effects of ethanol on the

relative dehydrogenase activities were concentration- and time-dependent, but otsB strain possessed a higher level over the control strain under the same condition (Fig. S6B). These findings proved that the superior biotransformation performance of the otsB strain was largely attributed to higher cell viability during exposure to systems containing increased ethanol and CA, which may arise from elevated trehalose accumulation.

### Protective mechanisms of trehalose on ethanol tolerance in *A. simplex*

Due to the strongest stress tolerance and superior biotransformation performance, the otsB strain accumulating the most trehalose was selected to investigate the protective mechanisms of trehalose. The protective mechanisms of trehalose in *A. simplex* when exposed to the two types of ethanol treatment were discussed, including long-term growth at 6% (vol/vol) of ethanol and short-term shock by 20% (vol/vol) of ethanol, respectively. As shown in Fig. 6a,

**Fig. 6** The trehalose content (a) membrane fluidity; (b) ROS level; (c) antioxidant enzymes activities (d) and ATP content; (e) in the control strain and the *otsB* overexpression *A. simplex* strain under different stress conditions. i, Growth in LB medium until late exponential phase; ii, growth in LB medium supplemented with 6% (vol/vol) ethanol until late exponential phase; iii, no stress shock for 1 h; iv, 20% (vol/vol) ethanol shock for 1 h; All data represent the mean values from three independent experiments



the trehalose amounts in the *otsB* and the control strains during exposure to different ethanol stresses were all enhanced, but the *otsB* strain always had a higher level of trehalose than the control strain.

The cell membrane is the primary target site of ethanol toxicity [38]. Without stress, the cell morphology of both strains was rod-like and had clear surfaces with regular creases and a nearly smooth cell body. After exposure to ethanol, the cell surface structure and morphology were remarkably altered. Additional wrinkle formation, several tiny pores appearance, depressed deformation, and the leakage of cellular debris were observed. However, the *otsB* strain maintained better cell integrity compared with the control strain, especially the outermost cell surface structure (Fig. S7). Water plays an important role in the structure of biological membranes. However, ethanol can substitute water, and thereby change the positioning of molecules on the membranes, influencing the interaction between lipids and proteins, and ultimately damaging membrane structure

and functions [39]. In yeasts, trehalose can stabilize the membrane against phase transitions by replacing water and ethanol and subsequently forming hydrogen bonds between the hydroxyl groups of trehalose with the polar head groups of lipids bilayer [4, 11, 40, 41], or by forming a glass-like structure (vitrification) to surround the proteins [42]. Trehalose is a small molecular-mass “chaperone” and can stabilize proteins in their native states during injury and suppress the aggregation of denatured proteins [42–44]. The similar protective role of trehalose on cell integrity has also been observed in yeast subjected to ethanol [5, 20]. As shown in Fig. 6b, the membrane fluidity of both strains was greatly elevated upon exposure to ethanol, but the *otsB* strain maintained a lower value than the control strain, indicating that trehalose accumulation can decrease the fluidizing effect of ethanol on the membrane of *A. simplex* cells. In summary, the maintenance of cell integrity and membrane stability mediated by trehalose may partly account for ethanol tolerance enhancement in *A. simplex*.

ROS derived from the ethanol exposure generated toxicity to cells. As shown in Fig. 6c, no obvious difference was found in the ROS levels between the *otsB* and control strains under non-stressed condition. Additional ROS was generated after ethanol treatment, but the level in the *otsB* strain was always lower than that in the control strain, indicating the former suffered from much less oxidative damage. The trehalose-induced ROS detoxification was further proven by analyzing the activities of antioxidant enzymes, including CAT, SOD, and GPx (Fig. 6d). The variations in the expression levels of the selected genes also corresponded well to the alterations in the specific activities for translated products and ROS content (data not shown). Trehalose accumulation protects yeasts from ethanol-induced ROS damage [7, 20, 45–47]. We speculated that trehalose might directly sense the cellular stress signal (such as ROS) mediated by ethanol and subsequently boost ROS scavenging capacity by activating antioxidant enzymes. This effect consequently increased cell viability during exposure to ethanol stress.

As an index of cell energetics, the cellular ATP levels in both strains were measured. No evident difference was found in ATP contents between the *otsB* and control strains under non-ethanol condition. During exposure to ethanol, an evident loss in ATP levels occurred, but the *otsB* strain maintained a higher level compared with the control strain (Fig. 6e). Results demonstrated that energy consumption was the crucial reason for the reduction in cell viability after ethanol treatment. Similar observations have been found in previous reports [48, 49]. Therefore, trehalose overproduction facilitated elevated energy generation in the form of ATP, which was beneficial for keeping sufficient energy to run all adaptive mechanisms, ultimately enhancing ethanol resistance.

In summary, the adaptive modifications of compatible solutes played a vital role in *A. simplex* under various ethanol stresses, where the amount of trehalose and glycerol increased. Meanwhile, the levels of glutamate and proline decreased regardless of long-term growth at mild concentration or short-term shock by a high concentration of ethanol treatment. By overexpression of three biosynthesis-related genes, more trehalose was accumulated in *A. simplex* and then functioned as an efficient protectant when cells were challenged with different stresses. The cellular trehalose amount was positively correlated with the levels of cell tolerance either to organic solvents or other abiotic stresses (such as oxidative and salt stresses). Interestingly, the horizontal introduction of trehalose biosynthesis genes from *A. simplex* to *E. coli* also effectively enhanced cell tolerance to various stresses, but the increased influence was closely linked to the type and level of stress. *A. simplex* engineering strains that accumulated more trehalose exhibited higher productivity due to better viability in systems containing more substrate and ethanol. Among them, the best *otsB* strain

showed good biotransformation performance in system V [35 g L<sup>-1</sup> CA dissolved in 10% (vol/vol) ethanol], whereas the control strain almost lost its capability. The protective mechanisms of trehalose against stresses were involved in maintaining cell integrity and membrane stability, alleviating oxidative damage, and satisfying energy requirements. Taken together, trehalose could protect *A. simplex* against various types of stress and its genetic modification for overproduction is effective for improving cell stress tolerance, which consequently leads to enhancement in biotransformation productivity.

**Acknowledgements** This work was financially supported by Natural Science Foundation of China (nos. 21978220), the Natural Science Foundation of Tianjin (nos. 18JCZDJC32500), Tianjin Technical Expert Project (nos. 19JCTPJC50800), the Open Fund of Ministry of Education Key Laboratory of Molecular Microbiology and Technology, Nankai University, Tianjin Municipal Science and Technology Commission (17PTGCCX00190), and 2019 Tianjin innovation and entrepreneurship training program for college students (national grade) (201910057051).

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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