Engineering prokaryotic regulator IrrE to enhance stress tolerance in budding yeast

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Abstract

Background: Stress tolerance is one of the important desired microbial traits for industrial bioprocess and global regulatory protein engineering is an efficient approach to improve strain tolerance. In our study, IrrE, a global regulatory protein from prokaryotic organism *Deinococcus radiodurans*, was engineered to confer yeast the improved tolerance to the inhibitors in lignocellulose hydrolysates or high temperature.

Results: Three IrrE mutants were developed through directed evolution and the expression of these mutants could improve the yeast fermentation rate by 3- to 4-fold in the presence of multiple inhibitors. Subsequently, the tolerance to multiple inhibitors of single-site mutants based on the mutations from the variants was then evaluated, and eleven mutants including L65P, I103T, E119V, L160F, P162S, M169V, V204A, R244G, Base 824 Deletion, V299A and A300V were identified to be critical for the improved FAP tolerance. Further studies indicated that IrrE caused genome-wide transcriptional perturbation in yeast, and the mutant I24 led to the rapid growth of *Saccharomyces cerevisiae* mainly by regulating the transcription level of transcription activators/factors, protecting the intracellular environment and enhancing the antioxidant capacity under inhibitor environment, which reflected the plasticity of IrrE. Meanwhile, we observed that the expression of the wild-type or mutant IrrE could also protect *Saccharomyces cerevisiae* from the damage caused by thermal stress. The recombinant yeast strains were able to grow with glucose at 42 ºC.

Conclusions: IrrE from *Deinococcus radiodurans* can be engineered as a tolerance-enhancer for *Saccharomyces cerevisiae*. Systematic research on the regulatory model and mechanism of a prokaryotic global regulatory factor IrrE to increase yeast tolerance provided valuable insights for the improvement of microbial tolerance to complex industrial stress conditions.

Background

Concerns about energy supplies and global climate change have led to a growing interest worldwide for producing biochemicals, biofuels, and biomaterials from lignocellulosic biomass[1, 2]. For the efficient fermentation of lignocellulosic hydrolysates, it is highly desired to improve strain tolerance to specific stresses, such as the inhibitors inevitably released from the pretreatment of lignocellulose biomass and the high temperature employed in the bioprocess of simultaneous saccharification and ethanol fermentation (SSF)[3, 4]. Studies on genetic analysis to stress tolerance have yielded several beneficial genetic traits associated with furfural or acetic acid tolerance[5–7]. The deletion or overexpression of some resistant structural genes has proven useful for improving the strain tolerance to a single inhibitor[8–10]. However, due to the diversity and complexity of inhibitors in lignocellulosic hydrolysates, the tolerance to combined inhibitors becomes more difficult and involves multigenic traits[11]. Thus, regulating multiple genes simultaneously may be a feasible route to enhance strain tolerance to complex inhibitors. Recently, Multilevel Defense System (MDS) has been developed to eliminate a variety of stresses and economically promote ethanol production from industrial yeast[12].

Global transcription machinery engineering (gTME) is a directed evolutionary method to improve cellular phenotype, and a strain library with different phenotypes was generated by using this method[13]. This approach has been successfully explored in reprogramming cellular tolerancing to substrates or products phenotypes[13–16]. For example, Alper and his co-workers generated a mutant library of the TATA binding protein Spt15 and conferred yeast increased ethanol tolerance[15]. In recent years, several global regulators in *Escherichia coli* have been well characterized, such as CRP, FNR, IHF, FIS, ArcA, NarL and Lrp, which directly regulate the expression of 51% genes in *E. coli*[17, 18]. Through the genetic analysis of a DNA damage-sensitive strain *Deinococcus radiodurans*, the regulatory protein IrrE was discovered[19]. The overexpression or modification of IrrE has been proved to be capable of enhancing the tolerance of *E. coli* to various stressful conditions, such as ethanol, butanol, salt, furfural, acetic acid, 5-hydroxymethyl-2-furaldehyde (HMF) and osmotic stress[20–24]. The expression of protein IrrE also protected *Zymomonas mobilis* against ethanol and acid stress[25]. The introduction of IrrE efficiently improved *Arthrobacter simplex* tolerance to organic solvents and various abiotic stresses as well[26]. Recently research exhibited that *Sphingomonas sp.* with the gene *IrrE* and *Pseudomonas putida* possessing the same gene showed enhanced tolerance to acid[27, 28]. Furthermore, the transcriptome and proteome analysis proved that IrrE did act as a global regulator in *E. coli* and *A. simplex* and therefore brought about efficient stress tolerances, for instance, tolerating salt and ethanol stress[20, 29–31]. However, the application and mechanism research of IrrE mainly focused on prokaryotes (such as *E. coli*). In recent years, the regulatory effect of IrrE has also been studied in plants and yeast[20, 32, 33]. Encouraged by the success of IrrE engineering in prokaryotic organisms, plants and yeast, here we reported an example of engineering IrrE in *Saccharomyces cerevisiae* for the tolerance toward stress condition (Fig. 1). Although the wild-type IrrE was not efficient for *S. cerevisiae*, the directed evolution of IrrE was successfully applied to significantly improve yeast resistance to the mixture of multiple lignocellulose-derived inhibitors (furfural, acetic acid and phenol) and thermal stress. The preliminary results of mechanism analysis showed that the heterologous expression of IrrE played a complex regulatory role, which provides a better choice for enhancing cell tolerance.

Results And Discussion

Expressing IrrE to enhance yeast tolerance to acetic acid

To study the effect of IrrE on the inhibitor tolerance of *S. cerevisiae*, we first evaluated the role of IrrE on the strain tolerance to acetic acid, a primary inhibitor in lignocellulose hydrolysates. In the presence of acetic acid, the strain BY4742/IrrE entered the exponential phase at about 30 h and reached the stationary phase at about 54 h with the maximal OD_{600} = 3.56. In comparison, the growth of control strain was recovered until 48 h and reached a maximum OD_{600} = 3.12 at 67 h (Additional file 2: Fig. S1). The results showed that the heterologous expression of IrrE slightly enhanced the ability of *S. cerevisiae* cells against acetic acid shock, but it did not confer strain desired acid tolerance. Besides, we observed that the growth ability of strain BY4742/IrrE was also slightly increased in the presence of the mixture of three inhibitors (furfural, acetic acid and phenol) (Fig. 2). Previous studies have shown that engineered IrrE can

enhance acetic acid tolerance of *Zymomonas mobilis* and yeast[25, 34]. Therefore, it is necessary to take some measures to engineer IrrE in *S. cerevisiae* to optimize strain FAP tolerance further.

Directed evolution of IrrE to improve strain tolerance

The directed evolution strategy has been widely used in protein modification and it showed that engineered IrrE conferred *E. coli* enhanced tolerances toward lignocellulosic hydrolysates inhibitors[22]. To further optimize strain tolerance to lignocellulose-derived inhibitors, especially the mixture of multiple inhibitors FAP, directed evolution was employed to modify IrrE in *S. cerevisiae*. As described in the part of methods, the *IrrE* library of about 10^5 mutants were generated. The transformants were initially selected in the 96-well plates based on strain growth in SC-Ura medium with FAP (Fig. 1). The growth rate of the isolated mutants was further verified in the tube and flask (Fig. 1).

Accordingly, in the first round of mutagenesis and selection, two mutants BY4742/I12 and BY4742/I24, were isolated. Compared to the strain BY4742/pRS416 and the strain BY4742/IrrE, the significant growth increase of the two mutants was observed in the presence of FAP (Fig. 2). Meanwhile, strain BY4742/I24 exhibited higher FAP tolerance than the strain BY4742/I12 (Fig. 2). To further improve performance, the mutant *I24* from strain BY4742/I24 was used as the template for the second round of directed evolution, and strain BY4742/I37 was obtained. Compared to the strain BY4742/I24, the growth ability of strain BY4742/I37 under FAP stress was also slightly improved (Fig. 2). The experiment reflected the potential role of IrrE in regulating the transcriptional level of genes in *S. cerevisiae*.

Evolved IrrE to improve strain tolerance to the mixture of multiple inhibitors

As the top level of the hierarchical regulatory network in microorganisms, the global regulators control strain phenotypes by regulating large numbers of genes expression. The engineering of global regulators has been proved to be a highly efficient approach conferring cells desired complex phenotypes in both prokaryotic and eukaryotic microbes[15, 35]. This study has evolved IrrE to improve strain tolerance to the mixture of multiple inhibitors. The fermentation ability of the IrrE mutants was then comparatively analyzed in the SC-Ura medium supplemented with or without multiple inhibitors (0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol). In the absence of multiple inhibitors, the heterologous expression of *IrrE* in *S. cerevisiae* slightly weakened strain fermentation. The strain BY4742/pRS416 finished the fermentation at about 15 h, while 8.5 g/L glucose was still left in the culture of strain BY4742/IrrE (Fig. 3a). A slight decrease in the biomass concentration, specific growth rate, glucose consumption rate, the final ethanol titer, ethanol productivity and ethanol yield were also observed in the strain BY4742/IrrE (Table 1). These results indicated the extra metabolic burden of expressing heterologous *IrrE* gene to *S. cerevisiae*. However, the mutation of IrrE significantly increased strain biomass yield, glucose consumption rate, specific growth rate, the final ethanol titer, ethanol productivity and ethanol yield (Fig. 3a, Table 1).

Table 1
Fermentation parameters for the strains carrying the improved IrrE in the presence and absence of FAP. Results represent the mean of duplicate experiments.

FAP	Strains	μ (h^{-1})	r_{glu} (g/L/h)	Ethanol titer (g/L)	r_{eth} (g/L/h)	Ethanol yield (%)
-	BY4742/pRS416	0.51	1.11	8.27	0.39	82.6
	BY4742/IrrE	0.45	0.95	7.97	0.38	79.6
	BY4742/I12	0.64	1.34	8.48	0.71	84.7
	BY4742/I24	0.69	1.34	8.62	0.72	86.1
	BY4742/I37	0.67	1.34	8.57	0.71	85.6
+	BY4742/pRS416	0.079	0.13	8.65	0.057	86.4
	BY4742/IrrE	0.075	0.17	8.27	0.054	82.6
	BY4742/I12	0.073	0.42	8.70	0.18	86.1
	BY4742/I24	0.089	0.48	8.79	0.18	87.0
	BY4742/I37	0.10	0.61	8.80	0.18	87.1

When the mixture of three inhibitors was added in SC-Ura medium, the fermentation performance of five strains was all significantly affected. The second-round mutant strain BY4742/I37 grew into the stationary phase and exhausted all the glucose at about 33 h (Fig. 3b). The first-round mutants BY4742/I12 and BY4742/I24 finished the fermentation within 48 h and 42 h respectively (Fig. 3b). In contrast, the fermentation of strain BY4742/IrrE was extended to approximately 115 h, while strain BY4742/pRS416 was still in the early exponential phase at that time and finished the fermentation until 153 h. The glucose consumption rate and the ethanol productivity were in parallel with the growth rate (Fig. 3b). The mutant BY4742/I37 exhibited the highest glucose consumption rate of 0.61 g/L/h, which was about 4.7-fold and 3.6-fold of the strain BY4742/pRS416 and strain BY4742/IrrE respectively (Table 1). The ethanol productivities of the three mutants were all 0.18 g/L/h, which were about 3.2-fold and 3.3-fold of the strain BY4742/pRS416 and strain BY4742/IrrE respectively (Table 1). The above data demonstrated that although the ethanol productivity of the mutants was markedly increased, the five strains generated similar ethanol yield in the presence of multiple inhibitors.

Sequence-based analysis of the mutants

Based on the above results, the great tolerance enhancement occurred after two rounds of error-prone PCR. Thus, the three IrrE mutants, I12, I24 and I37 screened from these two rounds were sequenced, and the resultant amino acid sequences were aligned with the wild type IrrE (Fig. 4a and 4b). Mutant I12 differed from IrrE at seven amino acid loci, while mutant I24 and mutant I37 had four mutations and nine mutations respectively. According to the mutations identified in I12, I24 and I37, single-site mutants were

generated to investigate their effect on yeast tolerance to multiple FAP (Fig. 4c). For the mutants from I12, I103T, S133R, P162S and V204A showed a negative impact on strain growth under FAP-free condition. When the multiple inhibitors were added, the growth of strain I103T, P162S and V204A exhibited the moderate improvement compared to that of strain BY4742/IrrE, and the mutation V299A and A300V significantly increased strain growth ability, while M74T and S133R had only a little effect on strain sensitivity to FAP. Compared to the mutant I12, A300V was more tolerant to FAP stress, while I103T, P162S and V299A appeared more sensitive. For the four mutants from I24, the growth of E119V and L160F was decreased compared to strain BY4742/IrrE in the FAP-free medium. Under the FAP stress condition, the growth of A52E was rarely affected compared to that of the strain BY4742/IrrE. In contrast, E119V, L160F and R244G all rescued strain growth with inhibitors. Meanwhile, these three mutants appeared more sensitive to FAP than the mutant I24, suggesting that these three mutants work synergistically. In addition to the same four mutants as I24 has, I37 has five additional mutants and their growth was all decreased compared to strain BY4742/IrrE in FAP-free medium. M169V, E271K and Base 824 Deletion mutants showed a little increased tolerance compared to the control strain under FAP stress conditions, while the mutant L65P showed a moderately enhanced inhibitor tolerance. The improved tolerance of strain I37 may be the result of the combined action of nine site mutations. Through this comprehensive analysis, eleven mutants of IrrE protein, including L65P, I103T, E119V, L160F, P162S, M169V, V204A, R244G, Base 824 Deletion, V299A and A300V, were identified with an essential role in yeast FAP tolerance. As the reported structure of *Deinococcus deserti* IrrE, three domains are identified, including one zinc peptidase-like domain in the N terminal domain, one helix-turn-helix motif in the middle domain, and one GAF-like domain in the C-terminal domain[36]. Based on the sequence alignment and the homology model, the domain boundaries of *D. radiodurans* IrrE were determined as follows: the N-terminal domain covering residues1-161; the middle domain covering residues 162–203; and the C-terminal domain covering residues 204–328[21]. Through the mutant analysis, Vujicic-Zagar et al. indicate that the first and third domains are also critical regions for radiotolerance in strain *D. deserti*[36]. In this work, except for P162S and M169V, other mutations identified in the strain BY4742/I12, BY4742/I24 and BY4742/I37 all presented at the first and third domains, suggesting the critical role of these two domains in regulating *S. cerevisiae* to tolerant FAP stress.

Uncovering the global perturbation generated by IrrE in *S. cerevisiae* in response to the mixture of multiple inhibitors

To study the regulation mechanism of IrrE in *S. cerevisiae* cells in response to the mixture of multiple inhibitors, transcriptome sequencing and metabolite analysis were carried out. We examined the profiles of the two strains under normal growth conditions to study the genetic background differences between strain BY4742/IrrE and strain BY4742/pRS416. Samples in the middle of the lag phase of both strains under multiple inhibitors (0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol) were collected for RNA-seq to compare the transcriptional profile changes between them. To identify genes related to IrrE

regulation in response to FAP, we focused on genes that displayed more than 2-fold (log_2foldchange > 1.0 and *p* value < 0.05) increase or decrease in treated BY4742/IrrE strain compared with treated BY4742/pRS416 strain but not in untreated BY4742/IrrE strain compared with untreated BY4742/pRS416 strain. Also, we tested changes of some intracellular metabolites to understand how they responded to FAP stress. IrrE may regulate the external defense and internal repair system to increase strain inhibitors tolerance.

Acetic acid, furfural, and phenol are the main components of inhibitors produced by lignocellulose pretreatment and have been proved to cause accumulation of reactive oxygen species (ROS) in yeast[10, 32]. As shown in Fig. 5a, the ROS level in untreated strain BY4742/IrrE was slightly lower than that in untreated strain BY4742/pRS416. However, the ROS level in strain BY4742/IrrE was reduced by about 61.4% compared to that in the control strain after exposure to FAP, despite the simultaneous increase of ROS levels in both strains. These results implied that the strain BY4742/IrrE suffered less ROS damage. The same protection role of IrrE was also verified in *E. coli* and *Brassica napus* under salt shock and in *E. coli* and *A. simplex* under ethanol shock[19, 20, 30, 37]. The transcription levels of the genes encoding enzymes related to ROS detoxification were upregulated in treated strain BY4742/IrrE (Additional file 1: Table S1). We further determined the activities of superoxide dismutases and catalases, and the results showed that these ROS scavenging enzymes exhibited higher activity in the strain BY4742/IrrE compared to strain BY4742/pRS416 in the presence of FAP (Fig. 5b and 5c). These results indicated that the IrrE might reduce the level of intracellular ROS by increasing the activities of the related enzymes.

NADPH is necessary for the process of furfural detoxification in *S. cerevisiae*[38]. Pentose phosphate pathway (PPP) is the primary pathway for the production of NADPH in cells and the transcriptome results showed that the PPP-related genes were upregulated in treated strain BY4742/IrrE (Fig. 6d), which is accordance with the higher tolerance to FAP of the strain BY4742/IrrE.

The gene *hxk1* and *err3* involved in the glycolysis pathway were upregulated in treated strain BY4742/IrrE, and the gene *hxk1* is encoding the rate-limiting enzyme HXK1 in this pathway (Fig. 6c). Glycolysis is an energy-producing pathway under anaerobic fermentation conditions and ATP is the main form of energy in yeast cells. The detoxification of inhibitors is an energy-consuming process for yeast, and enhancement of energy supply seems to be necessary for yeast to resist FAP damage. The strain BY4742/IrrE showed a higher ATP yield than the strain BY4742/pRS416 in the presence of FAP (Fig. 5d).

The expression of genes related to trehalose and glycogen biosynthesis was upregulated in strain BY4742/IrrE under FAP stress (Fig. 6a and 6b). Trehalose act as a storage factor and stress protectant in yeast cells. Yeast can synthesize glycogen in response to the stress conditions. In consonance with the transcriptome analysis, the metabolite analysis showed that the trehalose content of the strain BY4742/pRS416 was 20.0% higher than that of the strain BY4742/IrrE under non-stress conditions. In

comparison, the trehalose content of the strain BY4742/IrrE was boosted by 51.6% compared to that of the strain BY4742/pRS416 under FAP stress (Fig. 5e). In line with our results, the trehalose contents were significantly increased in *E. coli* strain with IrrE under osmotic or salt stress[23, 30]. To summarize, the accumulation of trehalose and glycogen was significantly involved in the mechanism of IrrE to improve strain tolerance. Moreover, the transcription of genes related to glycogen degradation was also upregulated (Fig. 6b), which suggests that the accumulated glycogen can be phosphorylated and then enter the glycolysis pathway to release energy.

Meanwhile, the transcriptome results showed that some sets of genes were also upregulated in treated strain BY4742/IrrE, including genes related to DNA repair, some transcription factors/activators, some genes encoding membrane proteins and transport proteins and some genes associated with the ribosome (Additional file 2: Table S4). These sets of genes should play essential roles in FAP stress resistance of strain with IrrE.

From the transcriptome results we can see that IrrE regulated 869 (($\log_2$foldchange > 1.0 and *p* value < 0.05) genes in *S. cerevisiae* (Additional file 2: Figure S4), which were much more than the number of genes changed by mutated transcription factor Spt15p(132) and Taf25-3 in the application of gTME in *S. cerevisiae* under the unstressed conditions and oxidation stress respectively[15, 39]. As illustrated by Fig. 7, the global transcriptional factor IrrE may switch on diverse defense systems in yeast to resist FAP stress. ROS detoxification plays a vital role in enhancing yeast tolerance by reducing oxidative damage caused by FAP. Accumulated glycogen and trehalose act as pressure protectors to enhance yeast tolerance. At the same time, energy is stored in glycogen and released in the form of ATP via the glycolysis pathway for the utilization of energy demand pathways such as substance transport. NADPH produced by PPP can be used as a cofactor that is essential for the detoxification of inhibitors. Although we have analyzed the regulatory network of IrrE protein in yeast in response to inhibitors, the specific genes or proteins regulated by IrrE are still unclear and need further study.

The transcriptional regulation of I24 in yeast

Through the directed evolution method, three mutant strains with improved tolerance were obtained. Here the mutant strain BY4742/I24 was selected as a model to perform transcriptomic analysis and compared the profile with that of the control strain BY4742/pRS416. We first examined the patterns of the two strains under normal growth conditions. The results of transcriptome analysis proved that the protein I24 might accelerate the strain BY4742/I24 growth by modulating genes related to ergosterol biosynthesis and energy metabolism (Fig. 8). Sterols are significant sections of the cytomembrane and are concerned with strain resistance to hydrophobic molecules[40]. From Fig. 8 we can see that most of the genes associated with ergosterol biosynthesis were upregulated. Rapid cell growth requires sufficient energy supply. As the regulatory mechanism of IrrE, the transcriptome results of I24 also showed that some genes involved in the glycolysis pathway were upregulated and genes related to branch pathways of synthetic amino acids are down-regulated (Fig. 8a).

Like the analytical method of the strain BY4742/IrrE, this mutant group was then performed background removal. The results of transcriptome analysis showed that the protein I24 might enhance the tolerance of the strain BY4742/I24 by modulating genes in many aspects. Pma1 is necessary for maintaining cytosolic pH homeostasis and the electrochemical potential at the plasma membrane[41–43], and its encoding gene PMA1 achieved 2.56-fold time increase in treated strain BY4742/I24. The addition of amino acids enhances the tolerance of *S. cerevisiae* to ethanol and multiple inhibitors. In the meantime, the transcription levels of some amino acid permeases were also shown to be upregulated (Additional file 2: Table S5), which is in accordance with the higher tolerance to FAP of the strain BY4742/I24. Ribosome biogenesis is the core process of cell growth[44]. Many upregulated genes were enriched in the ribosome biogenesis set, which demonstrated that the rapid ribosome assembly in treated strain BY4742/I24 is to accommodate the protein processing required for rapid growth (Additional file 2: Figure S2). Furthermore, the protein I24 also enhanced strain tolerance by modulating some translation initiation factors and general stress response elements. Additional file 2: Table S5 showed that three transcription activators and two transcription factors were upregulated in treated strain BY4742/I24, among which MSN2 and MSN4 are two associated transcription activators and become active under many stress conditions[45, 46].

Therefore, the protein I24 may cause a wide range of perturbations by regulating the transcription level of transcription activators/factors, stabilizing the cell membrane and removing excess H^+ to protect the intracellular environment, and enhancing the antioxidant capacity under inhibitor environment. It suggested that on one hand, directed evolution conferred the strain BY4742/I24 the different regulatory mechanisms; on the other hand, it reflected the plasticity of IrrE.

The effect of IrrE on strain thermotolerance

To explore whether the global regulator IrrE from prokaryotes could elicit the tolerance of yeast cells to other industrially relevant stress, the effect of the wild and mutant IrrE on strain thermotolerance was investigated. As shown in Fig. 9, the fermentation ability of the five strains was compared on growth, glucose consumption and ethanol production in SC-Ura medium at 38 ºC and 42 ºC. Under the heat stock of 38 ºC, all the strains were capable of exhausting glucose in the fermentation medium. Notably, the strains expressing wild or mutant IrrE exhibited higher specific growth rate, glucose consumption rate, the final ethanol titer, ethanol productivity and final biomass concentration than the control strain BY4742/pRS416 (Fig. 9a and Table 2). At 42 ºC, the cell growth, sugar consumption, the final ethanol titer, ethanol productivity and biomass production were all significantly affected no matter in the control strain or the four recombinant strains (Fig. 9b and Table 2). The growth of the control strain BY4742/pRS416 was almost completely suppressed. It had approximately 5 g/L glucose consumed and 1 g/L ethanol produced within 35 h, and subsequently no more glucose was utilized until 72 h. However, excellent fermentation advantage was observed in recombinant strains. The mutant strain BY4742/I24 and BY4742/I37 could grow into the stationary phase at about 21 h and approximate 15.5 g/L glucose was consumed in 45 h. The strain BY4742/I37 exhibited a moderate advantage in the maximum biomass, the final ethanol titer, ethanol productivity and the final ethanol yield than those in strain

BY4742/I24. The strain BY4742/IrrE and BY4742/I12 exhausted 20 g/L glucose in 21 h and entered the stationary phase with the higher biomass and ethanol production. These results suggested that *S. cerevisi*ae cells could be conferred enhanced tolerance against thermal stress through expressing IrrE or its mutants, which was consistent with the previous findings that IrrE could protect *E. coli* cells from thermal shocks[20].

Table 2
Fermentation parameters for the strains harboring the improved IrrE at different cultivation temperature. Results represent the mean of duplicate experiments.

FAP	Strains	μ (h^{-1})	r_{glu} (g/L/h)	Ethanol titer (g/L)	r_{eth} (g/L/h)	Ethanol yield (%)
38 ºC	BY4742/pRS416	0.44	1.32	8.52	0.57	85.0
	BY4742/IrrE	0.56	1.5	8.73	0.58	87.1
	BY4742/I12	0.78	1.64	8.84	0.74	88.3
	BY4742/I24	0.73	1.65	8.90	0.74	88.9
	BY4742/I37	0.76	1.66	8.76	0.73	87.5
42 ºC	BY4742/pRS416	0.03	0.21	1.33	0.038	13.3
	BY4742/IrrE	0.162	0.89	8.34	0.14	83.3
	BY4742/I12	0.163	0.92	8.90	0.15	88.9
	BY4742/I24	0.106	0.32	6.26	0.11	62.5
	BY4742/I37	0.119	0.34	7.05	0.12	70.4

We further evaluated the fermentation performance of the recombinant strains with the wild or mutant IrrE in the presence of multiple inhibitors under high temperature conditions. When the cultivation temperature was 38 ºC, the addition of FAP completely suppressed the growth of all five strains, which were still in the lag phase until 170 h. Rare glucose was consumed and little ethanol was produced (Additional file 2: Figure S3). Under the condition of 34 ºC, all strains exhibited better growth than those under the condition of 30 ºC in the absence of FAP (Fig. 10). Similarly, the biomass yield, the final ethanol titer, ethanol productivity, glucose consumption rate and specific growth rate of the three mutants was significantly increased compared to the strain BY4742/pRS416, while the slight metabolic burden was observed in strain BY4742/IrrE (Fig. 10). However, at 34 ºC, the presence of FAP remarkably enlarged the lag phase and reduced the glucose consumption rate and the ethanol productivity. The growth of strain BY4742/pRS416 was still in the lag phase until 170 h, while the strain BY4742/IrrE entered the exponential phase at approximately 135 h (Fig. 10). The strain BY4742/I37, which exhibited the best fermentation capacity, could grow into the stationary phase at about 80 h. The strain BY4742/I12 and

BY4742/I24 finished the fermentation at about 125 h and 100 h respectively (Fig. 10). The glucose consumption rate and ethanol productivity were consistent with the strain growth (Fig. 10).

We found that the strain BY4742/IrrE exhibited better performance compared with the mutant strains with higher tolerance to FAP stress, suggesting that the mechanism referred to cellular tolerance to FAP differs from that for resistance to thermal stress. Meanwhile, the different stresses have a synergistically negative effect on cell survival. The increase of fermentation temperature made yeast cells face more severe challenges under the same concentration of FAP. The synergy of temperature and inhibitors was also observed during simultaneous saccharification and co-fermentation of pretreated corn stover[47]. However, the engineering of IrrE could achieve the purpose of enhancing the tolerance of yeast cells to two complex phenotypes (FAP tolerance and thermal tolerance) simultaneously. Further studies are ongoing to clarify the molecular mechanisms by which IrrE confers improved thermal tolerance in *S. cerevisiae*.

Conclusions

In our study, the engineering of global regulatory protein was carried out to improve the tolerance of yeast cells to the multiple inhibitors existed in lignocellulose hydrolysates and high temperature. Through the directed evolution of the regulator IrrE from *D. radiodurans* in *S. cerevisiae*, three IrrE mutants with much higher FAP tolerance were developed. The sequence analysis revealed that eleven locus mutations including L65P, I103T, E119V, L160F, P162S, M169V, V204A, R244G, Base 824 Deletion, V299A and A300V were critical for the enhanced FAP tolerance. We further carried out transcriptome and metabolome analysis and found that IrrE caused global perturbation in *S. cerevisiae* by regulating diverse defense systems to resist FAP stress, including ROS detoxification, NADPH supply, DNA repair, transcription factors/activators, membrane proteins and transport proteins, ribosome proteins, the accumulation of ATP, glycogen and trehalose. Besides, we also compared the differences between the transcriptomes of I24 in the presence and absence of inhibitors and found that I24 led to the rapid growth of *S. cerevisiae* mainly by regulating the transcription level of transcription activators/factors, protecting the intracellular environment and enhancing the antioxidant capacity under inhibitor environment. Meanwhile, we observed that the expression of the wild type or mutant IrrE could also protect *S. cerevisiae* from resisting thermal stress. Systematic exploration of the global regulation in yeast with a prokaryotic global regulatory factor IrrE puts forward valuable insights for the improvement of microbial tolerance to complex industrial stress conditions.

Methods

Strains and plasmids

Strains used in this study are shown in Table 3. *S. cerevisiae* BY4742 (MATα, his3, leu2, lys2, and ura3) was used for constructing the recombinant strains. *E. coli* DH5α was used for gene cloning and plasmid construction.

To construct the wild-type plasmid pRS416-HXT7p-IrrE-TEF1t, the HXT7 promoter and TEF1 terminator were amplified from *S. cerevisiae S288C* respectively using primers HXT7-F/HXT7-R and TEF1t-F/TEF1t-R. The HXT7 promoter was digested with BamHI and EcoRI, and the TEF1 terminator was digested with SalI and XhoI. The gene *IrrE* from *D. radiodurans* was reconstructed by PCR to match the codon preference of *S. cerevisiae* using primers IrrE-F/IrrE-R. After purification and digestion with EcoRI and SalI, the gene *IrrE* was ligated into the pRS416 vector with HXT7 promoter and TEF1t terminator respectively to yield plasmid pRS416-HXT7p- IrrE-TEF1t (Additional file 2: Figure S7). The primers used for plasmid construction were shown in Additional file 2: Table S2.

Media and culture condition

Yeast strains were cultivated in liquid SC-Ura medium (synthetic complete medium without uracil; 6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose, 0.1 g/L leucine, 0.02 g/L histidine, and 0.02 g/L tryptophan). Luria-Bertani medium (10 g/L peptone, 5 g/L yeast extract, and 5 g/L sodium chloride) containing 100 mg/L ampicillin was used to cultivate *E. coli.*

Directed evolution of IrrE

The mutant library of the gene *IrrE* was constructed by error-prone PCR, and transformed into yeast strain BY4742 for screening the mutants with improved inhibitor tolerance. Plasmid pRS416-HXT7p-IrrE-TEF1t was used as the template for the first-generation mutagenesis. Twenty nanograms of DNA template was added to a solution containing 7.0 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dGTP, 0.5 mM dTTP, 0.5 mM dCTP, 1.0 μM primer IrrE-F, 1.0 μM primer IrrE-R, 1 μl of Taq DNA polymerase (5U/μl), 0.35 mM $MnCl_2$, and distilled water to make the final volume 100 μl. The error-prone PCR products were purified, digested with EcoRI and SalI and inserted into the linearized plasmid pRS416-HXT7p-IrrE-TEF1t digested by the same restriction endonuclease to replace the wild-type *IrrE* gene. The ligation products were transformed into *S. cerevisiae* BY4742 using the lithium acetate/single-stranded carrier DNA/PEG method, and then plated on SC-Ura-agar plates for selection. The library size was approximately 10^5. Subsequently, approximately 40 groups of screening by 96-well plates with SC-Ura medium containing 0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol have been conducted, and 60 strains with shorter lag period of the growth curve compared with BY4742/pRS416 strain were obtained to perform tube and flasks fermentation respectively. According to the length of the lag period of the growth curve and the final OD_{600} of the mutant strains against BY4742/pRS416 strain in inhibitor environment, the mutant strain BY4742/I12 and strain BY4742/I24 were selected. The process was then repeated for mutant IrrE in the second round of mutagenesis. To make sure that enhanced tolerance is a consequence of the mutated genes of *IrrE*, the plasmids containing the mutated *IrrE* were isolated and retransformed.

Site-directed and insertional mutagenesis

Single-site mutants (A52E, L57P, L65P, M74T, I103T, S133R, E119V, L160F, P162S, M169V, V204A, R244G, E271K, Base 824 Deletion, V299A, A300V) were generated from pRS416-HXT7p-IrrE-TEF1t through two-step PCR procedure to replace wild codons through an overlap extension-PCR protocol. Using the process of constructing the I103T mutant as an example, step one, two simultaneous PCR reactions were carried out. One fragment was amplified using the primers IrrE-F/I130T-R and the other PCR reaction was performed with a primer pair that included I130T-F and IrrE-R. Then the two fragments were used as the templates and overlapped during the second PCR reaction to obtain full-length mutated IrrE. After gel purification and digestion, the mutated *IrrE* gene was cloned into EcoRI and SalI sites of plasmid pRS416-HXT7p-IrrE-TEF1t replacing the wild-type *IrrE* gene. The ligation products carried the mutated *IrrE* genes were then transformed into *E. coli* DH5α competent cells to obtain the corresponding plasmids. Sequence these plasmids to ensure that the correct mutanted genes were obtained. The purified plasmids were then transformed into *S. cerevisiae* BY4742 to get the corresponding mutants.

Growth assays

A single colony of yeast strains was first pre-cultured at 30 ºC and 220 rpm for 20 h in SC-Ura medium. Then, the pre-cultures were transferred into 100 mL SC-Ura medium with an initial OD_{600} of 0.1, incubating for 20–24 h at 200 rpm and 30 ºC to get the seed cultures. To test the effect of the IrrE module on strain inhibitor tolerance, the seed cultures of the strain BY4742/IrrE and the control strain BY4742/pRS416 were cultivated in SC-Ura medium (the initial OD_{600} was approximately 0.2) added with 3.2 g/L acetic acid in 250 mL flasks with a working volume of 100 mL at 150 rpm and 30 ºC. To test the inhibitor tolerance of the mutants, the seed cultures prepared as the description above were diluted to an optical density at 600 nm of 0.1, and 5 µl aliquots of a 10-fold dilution series were spotted onto SC-Ura agar plates supplemented with or without 0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol. The plates were incubated at 30 °C for three days. To test the thermotolerance of mutants, the plates without inhibitors were incubated at 42 °C for three days.

Fermentation

The seed cultures of the control stain BY4742/pRS416, strain BY4742/IrrE and the mutants were prepared as the description above. The fermentation ability of yeast strains in the presence of multiple inhibitors was comparatively analyzed. The fermentation was performed in SC-Ura medium in 250 mL flasks with a working volume of 100 mL at 30 °C and 150 rpm. The SC-Ura medium was supplemented with or without multiple inhibitors – 0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol. To test the fermentation ability of yeast strains at different temperatures, the seed cultures were incubated in 100 mL SC-Ura medium at 150 rpm. The fermentation temperature was maintained at 30 °C, 34 °C, 38 °C and 42 °C. The initial cell densities of fermentation were adjusted to OD_{600} of approximately 0.2. Rubber stoppers were used to cap the flasks to maintain microaerophilic conditions.

Transcriptome analysis of *S. cerevisiae* strains by RNA-seq

The seed cultures of the control stain BY4742/pRS416, strain BY4742/IrrE and strain BY4742/I24 were prepared as the description above and then were inoculated in SC-Ura medium supplemented with or without multiple inhibitors – 0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol, with an initial OD_{600} of 0.2. The cells were grown until the middle of the lag phase at 30 °C in a fermenter (150 rpm) and after that, harvested to perform total RNA isolating using Trizol and was quantified and qualified by Agilent 2100 Bioanalyzer. Each sample had three biological replicates. The library was generated using NEBNext® Ultra™ RNA Library Prep Kit (Illumina, NEB, USA), and Novogene Inc. conducted sequencing on Illumina HiSeq platform. Clean data (clean reads) were gained from raw reads (raw data) of fastq format through in-house perl scripts and then used in the downstream analyses. The data was normalized through Htseq software by using RPKM (Reads per Kilo bases per Million reads)-based normalization algorithm[48]. DESeq software was used to identify differentially expressed genes with $\log_2$foldchange > 1.0 and p value < 0.05. Significantly enriched GO (Gene Ontology) terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were selected through compared with the reference genome with a threshold of p value < 0.05. The gene information was found on Saccharomyces genome database (SGD) [49].

Reactive oxygen species analysis

ROS content was detected by DCFH-DA staining method[50] with some modifications. Specifically, cells were resuspended in phosphate buffer (PBS, pH = 7.0) with a final concentration of 10^7 cells/mL after washing twice with PBS. Then, adding 10 μg of DCFH-DA (using a 2.5 mg/ml stock dissolved in DMSO) to 1 mL of cell suspension and incubated for 60 min at 30 °C, after which cells were washed twice and resuspended in 1 mL PBS. The relative fluorescence intensity was measured in a multimode plate reader (SpectraMax M2, Molecular Devices, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The OD_{600} value of cell suspension was also measured.

Antioxidant Enzyme Activity, Trehalose and ATP Determination in *S. cerevisiae* strains

After collected, cells were washed and resuspended in PBS buffer. Then the SOD was analyzed by using the superoxide dismutase (SOD) assay kit (WST-1 method) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The catalase (CAT) activity was detected by using the CAT assay kit (Beyotime Biotechnology, Shanghai, China).). The ATP content was measured by using an ATP assay kit (Beyotime Biotechnology, Shanghai, China). The protein concentration was measured using the Bicinchonininc acid (BCA) protein assay kit (Dingguo Changsheng Biotechnology Co., LTD, Beijing, China) to adjust the activity of enzymes and the content of ATP. The intracellular trehalose was extracted and determined by a trehalose content detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Analytical methods

Spectrophotometer (TU-1810, Beijing, China) was used to measure OD_{600} to monitor cell growth. Glucose and ethanol concentrations were determined by high performance liquid chromatography (HPLC) using an Aminex HPX-87H ion-exchange column (Bio-Rad, Hercules, CA, USA). The samples were filtered through a 0.2 μM filter before injecting it into the HPLC system. The column was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min at 65 ºC, and detection was performed with the Waters 2414 refractive index detector.

Abbreviations

S. cerevisiae: *Saccharomyces cerevisiae*; *E. coli: Escherichia coli; D. radiodurans*: *Deinococcus radiodurans*; FAP: furfural, acetic acid and phenol; SSF: simultaneous saccharification and ethanol fermentation; HMF: 5-hydroxymethyl-2-furaldehyde; SC: synthetic complete medium; SC-Ura: SC medium without Uracil; DEGs: differentially expressed genes; ROS: reactive oxygen species; SOD: superoxide dismutase; CAT: catalases; PPP: pentose phosphate pathway; GPX: glutathione peroxidase; DCFH-DA: 2’7’-dichlorofluorescein diacetate; PBS: phosphate buffer.

Declarations

Acknowledgments

Not applicable.

Authors’ contributions

LW, XW, YJY and BZL conceived and designed the study. LW and XW performed the directed evolution, selection, and fermentation experiment. LW, SJZ and ZQH carried out the construction of strains and data analysis. The manuscript was written with the contributions of all authors. All authors have read and approved the final manuscript.

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Availability of data and materials

The datasets generated during this study are included in this published article and its additional file.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Information

Additional file 1:

Table S1: Outline of the differentially expressed genes in the strain BY4742/IrrE and the strain BY4742/I24 relative to the control strain with or without FAP. $\log_2$foldchange>1.0 and *p* value<0.05.

Additional file 2:

Table S2: Yeast strains and plasmids used in this study.

Table S3: Primers used in this work with endonuclease restriction sites underlined and italicized as essential.

Table S4: Upregulated genes related to FAP stress response in the strain BY4742/IrrE.

Table S5: Upregulated genes related to FAP stress response in the strain BY4742/I24.

Figure S1. The growth behaviors of strain BY4742/pRS416 and BY4742/IrrE under acetic acid condition.

Figure S2. Transcriptional profiles of ribosome biogenesis by expressing I24. The strain BY4742/I24 and control strain BY4742/pRS416 were cultured in SC-Ura medium with FAP tolerance. Samples were taken in the middle of the lag phase. Box number exhibits transcriptional change, which is the foldchange of the transcriptional level of the strain BY4742/I24 to that of the control strain BY4742/pRS416. Upregulated genes were highlighted in red.

Figure S3. The growth behaviors, glucose consumption and ethanol production of strain BY4742/pRS416, BY4742/IrrE, BY4742/I12, BY4742/I24 and BY4742/I37 at 38 ºC in the presence of 0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol.

Figure S4. DEGs in the strain BY4742/IrrE in the presence of 0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol. $\log_2$foldchange>1.0 and *p* value<0.05.

Figure S5. Representation of differentially expressed genes in selected Gene Ontology (GO) categories and KEGG pathways in the strain BY4742/IrrE after exposure to multiple inhibitors (0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol) until the middle of the lag phase. (a) The most enriched GO terms of the upregulated genes. (b) The statistics of pathway enrichment of the upregulated genes. (c) The most enriched GO terms of the downregulated genes. (d) The statistics of pathway enrichment of the downregulated genes.

Figure S6. Representation of differentially expressed genes in selected Gene Ontology (GO) categories and KEGG pathways in the strain BY4742/I24 after exposure to multiple inhibitors (0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol) until the middle of the lag phase. (a) The most enriched GO terms of the upregulated genes. (b) The statistics of pathway enrichment of the upregulated genes. (c) The most enriched GO terms of the downregulated genes. (d) The statistics of pathway enrichment of the downregulated genes.

Figure S7. Plasmid map for plasmid pRS416-HXT7p-IrrE-TEF1t and IrrE library construction.

Figures

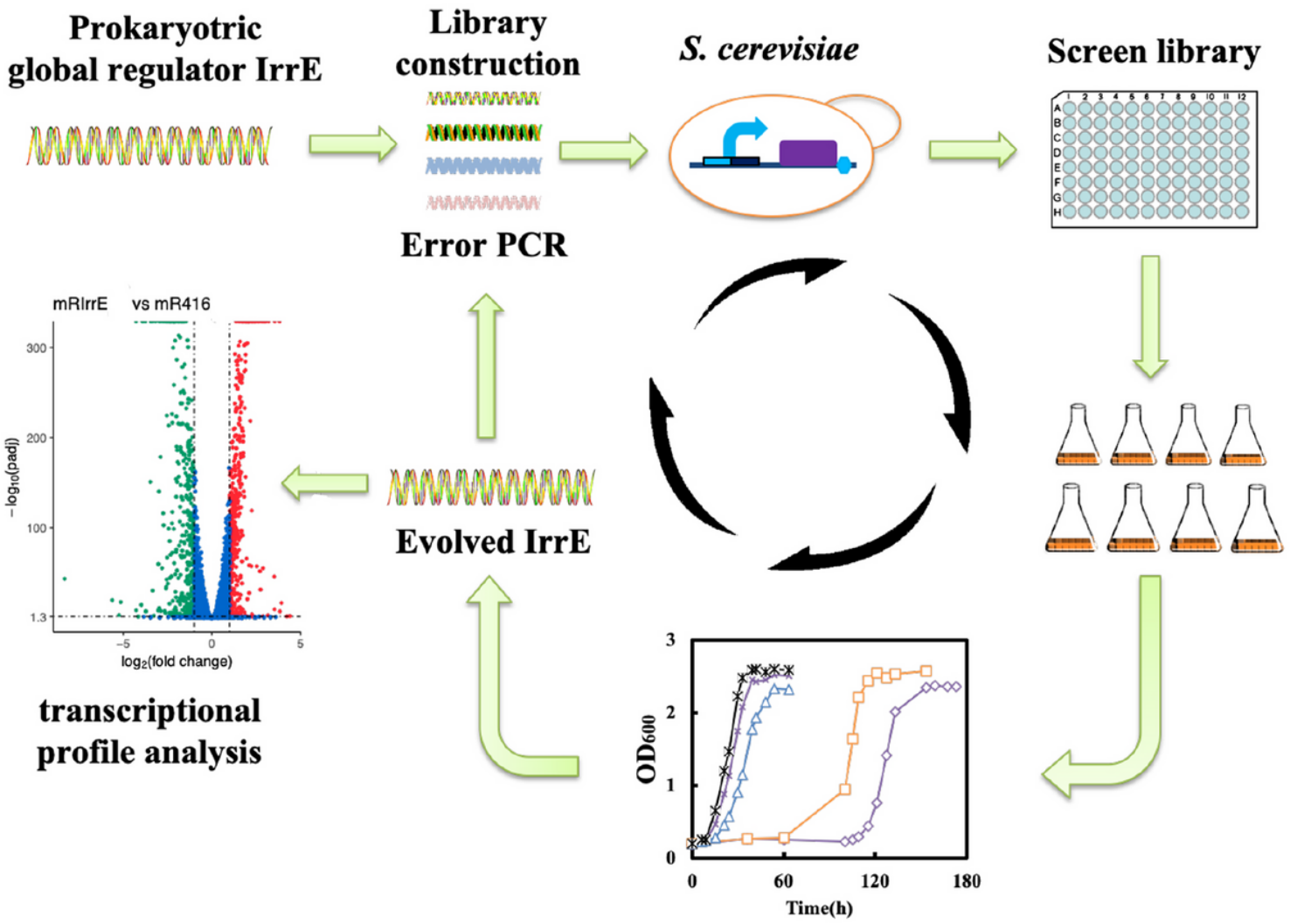

Figure 1

A general schematic for the directed evolution of IrrE to improve microbial tolerance. The strategy of error-prone PCR was carried out to introduce diversity to the genes. The mutant genes are then ligated into the pRS416 vector with HXT7 promoter and TEF1 terminator and transferred into S. cerevisiae BY4742. The library is screened with a high-throughput method based on the growth biomass in the presence of FAP. High biomass is related with faster growth and selected for further analysis in flask fermentation. Improved mutants are isolated for the next round of modification and screening. According to the transcriptome analysis, regulatory networks were constructed to reveal the regulatory mechanisms by which IrrE and I24 enhance yeast tolerance.

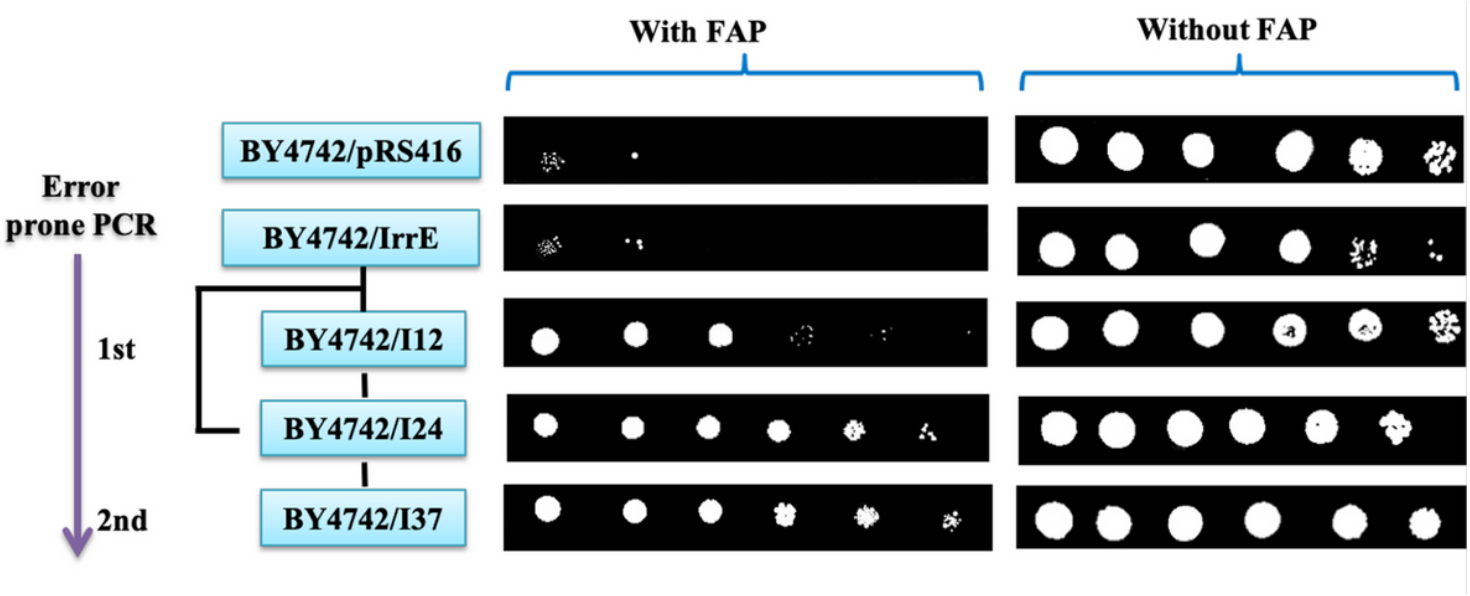

Figure 2

Effect of mutant IrrE on the FAP tolerance of yeast cells. The growth phenotypes of strain BY4742/pRS416, BY4742/IrrE, BY4742/I12, BY4742/I24 and BY4742/I37 were evaluated on SC-Ura plates in the absence and presence of 0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol.

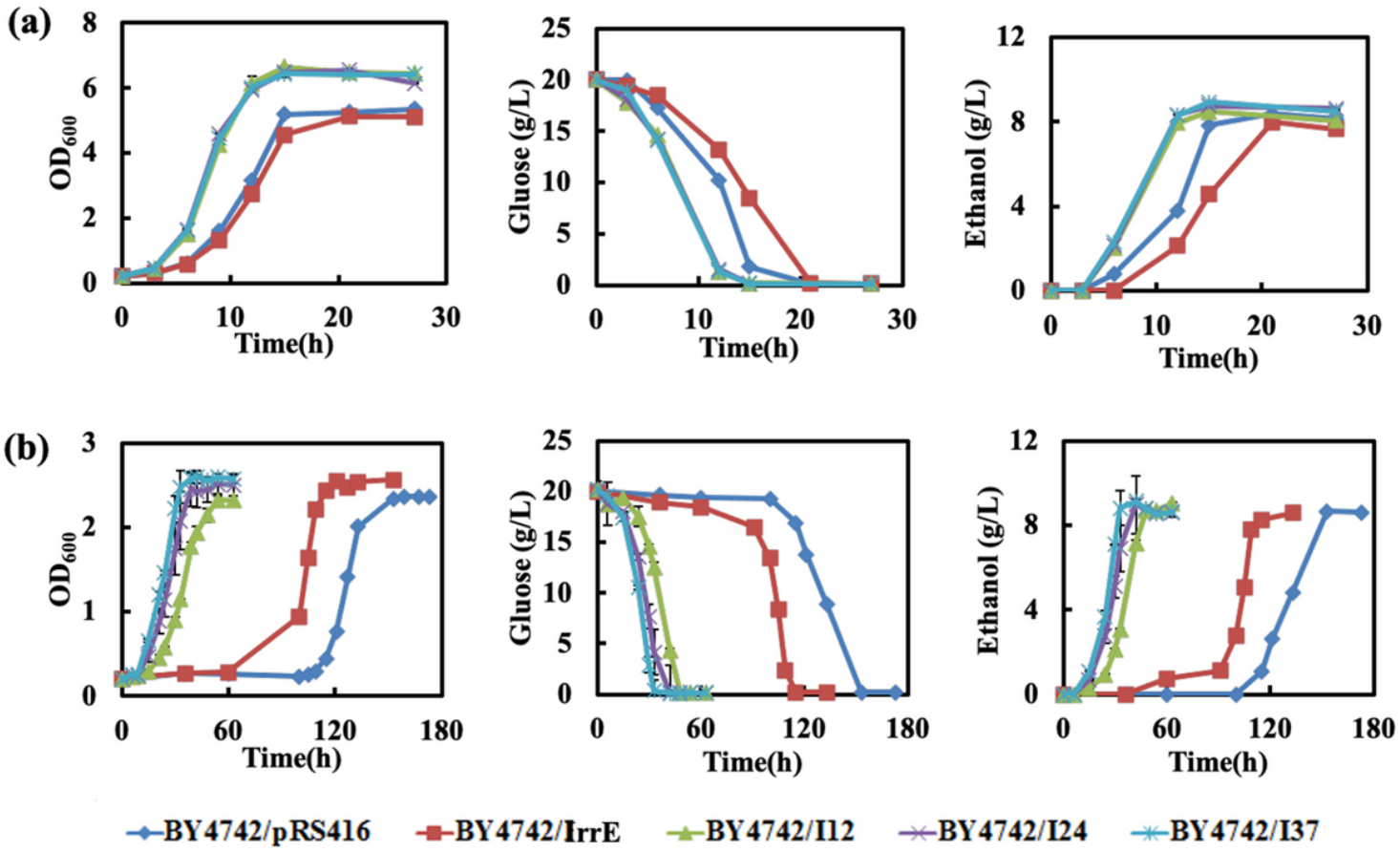

Figure 3

The effect of FAP on fermentation profiles of the strain BY4742/pRS416, BY4742/IrrE, BY4742/I12, BY4742/I24 and BY4742/I37. (a) The growth behaviors, glucose consumption and ethanol production of the strains in the absence of multiple inhibitors. (b) The growth behaviors, glucose consumption and ethanol production of the strains in the presence of 0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol. Results are the mean of duplicate experiments and error bars indicate s.d.

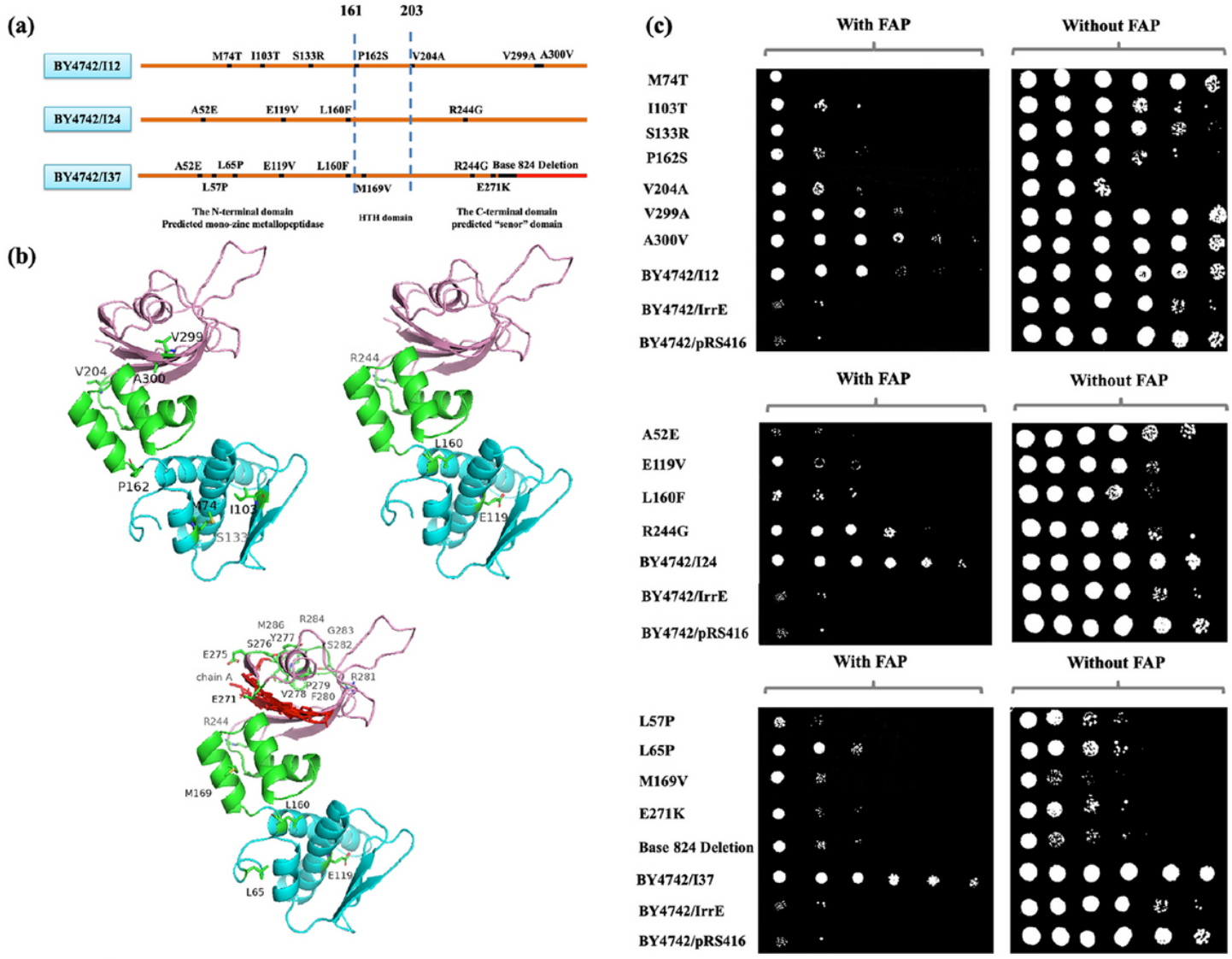

Figure 4

The effect of the single mutation sites on FAP tolerance. (a) The mutation sites of the mutants BY4742/I12, BY4742/I24 and BY4742/I37. (b) Exhibition of mutants BY4742/I12, BY4742/I24 and BY4742/I37 mutation sites in an IrrE modeled structure from D. radiodurans. IrrE domains: N- terminal domain colored in cyan; HTH domain colored in green; C-terminal domain colored in pink. (c) The growth phenotypes on SC-Ura plates of S. cerevisiae strain BY4742 expressing IrrE variants. The strains were cultivated on SC-Ura plates supplemented without or with 0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol. The experiments were repeated twice.

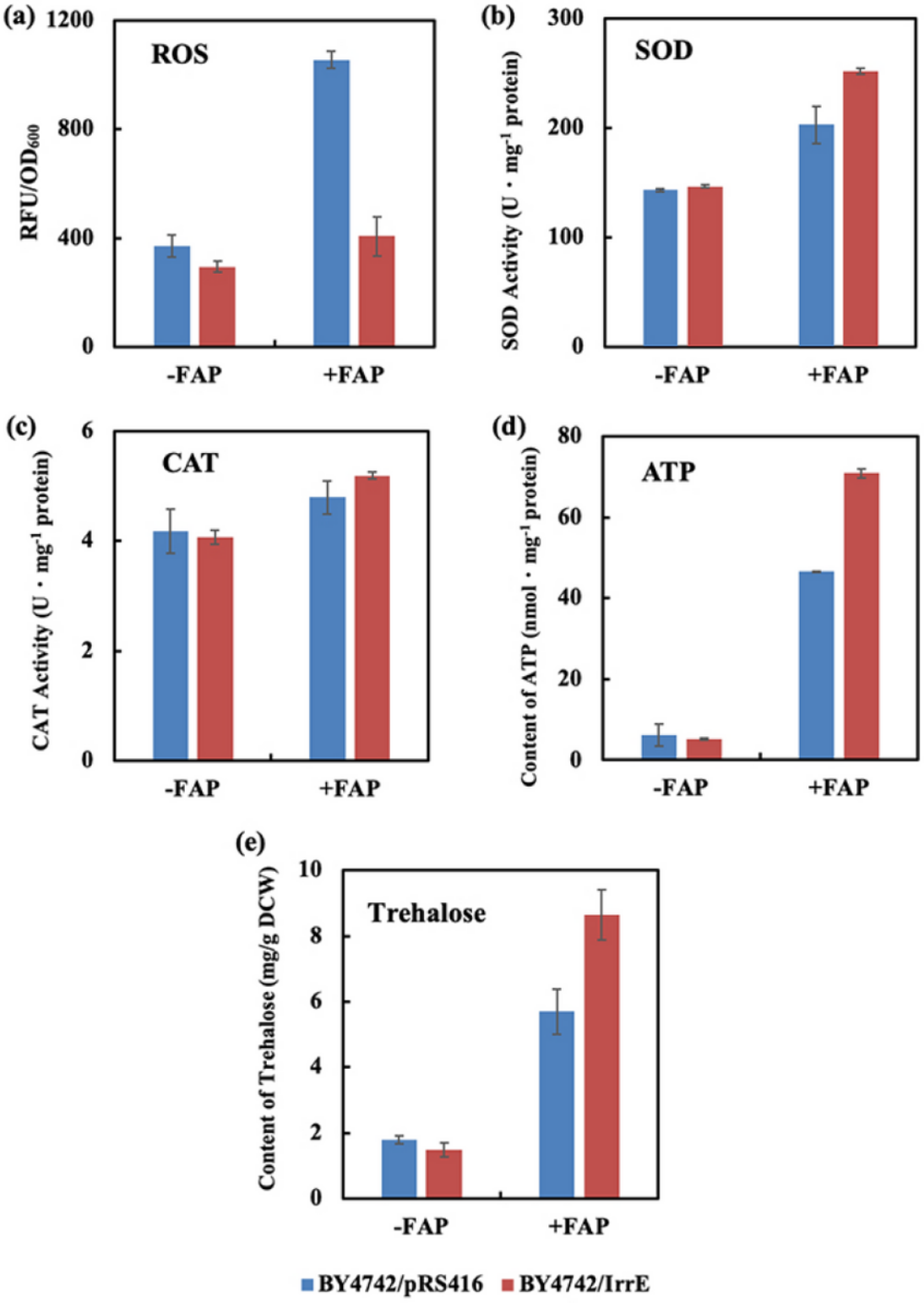

Figure 5

Effect of IrrE on the tolerance related metabolites in S. cerevisiae. Intracellular reactive oxygen species (ROS) content (a), the activities of SOD (b) and CAT (c), the intracellular ATP content (d) and trehalose content (e) in S. cerevisiae strains in their middle of the lag phase with or without FAP conditions. Results are the mean of duplicate experiments and error bars indicate s.d.

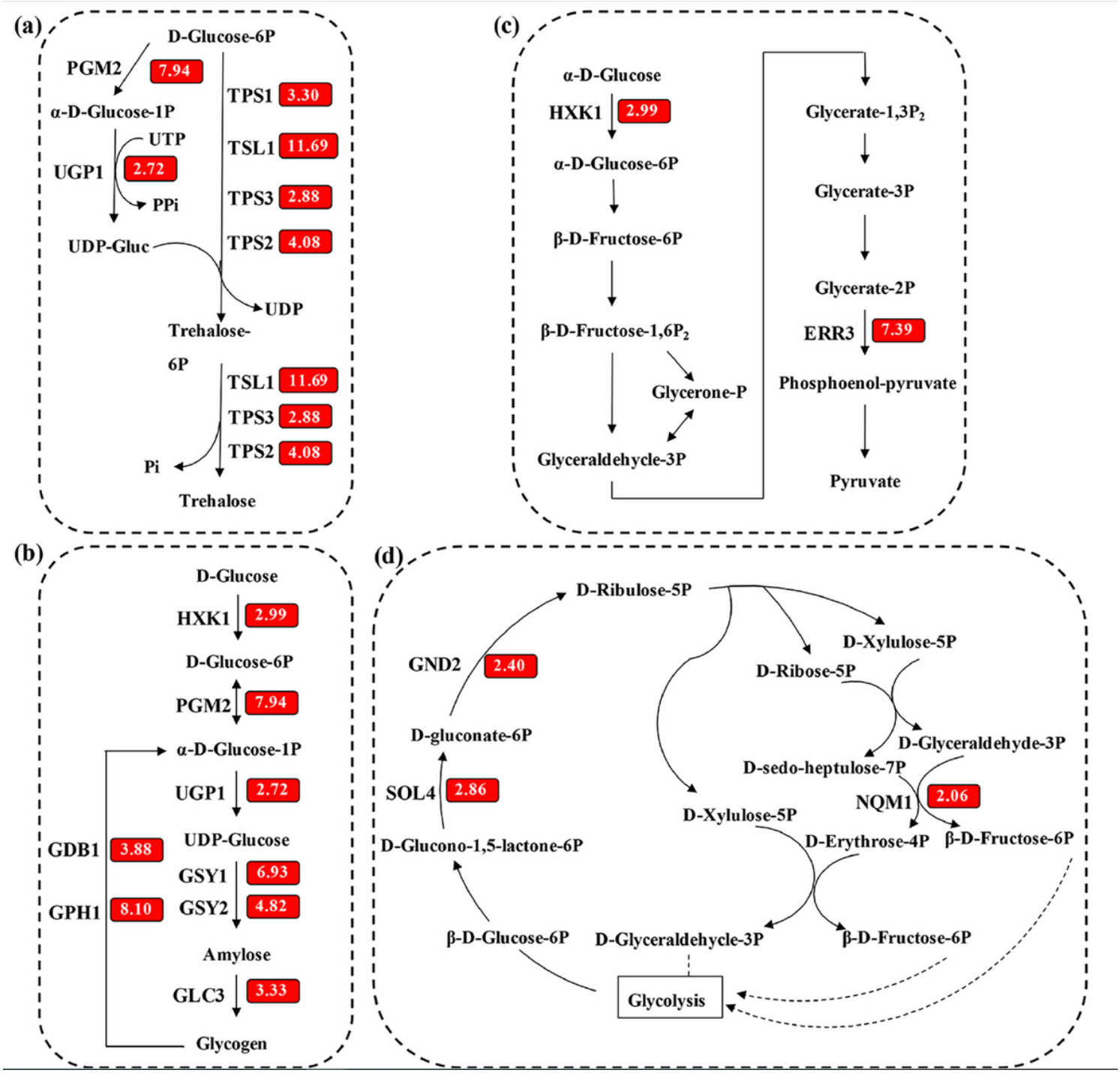

Figure 6

Effect of IrrE on yeast transcription. Transcriptional profiles of accumulation of pressure protectors (a and b) and energy (b), energy metabolism (c) and NADPH supply (d) by expressing IrrE. The strain BY4742/IrrE and control strain BY4742/pRS416 were cultured in SC-Ura medium with FAP tolerance. Samples were taken in the middle of the lag phase. Box number exhibits transcriptional change, which is the foldchange of the transcriptional level of the strain BY4742/IrrE to that of the control strain BY4742/pRS416. Upregulated genes were highlighted in red.

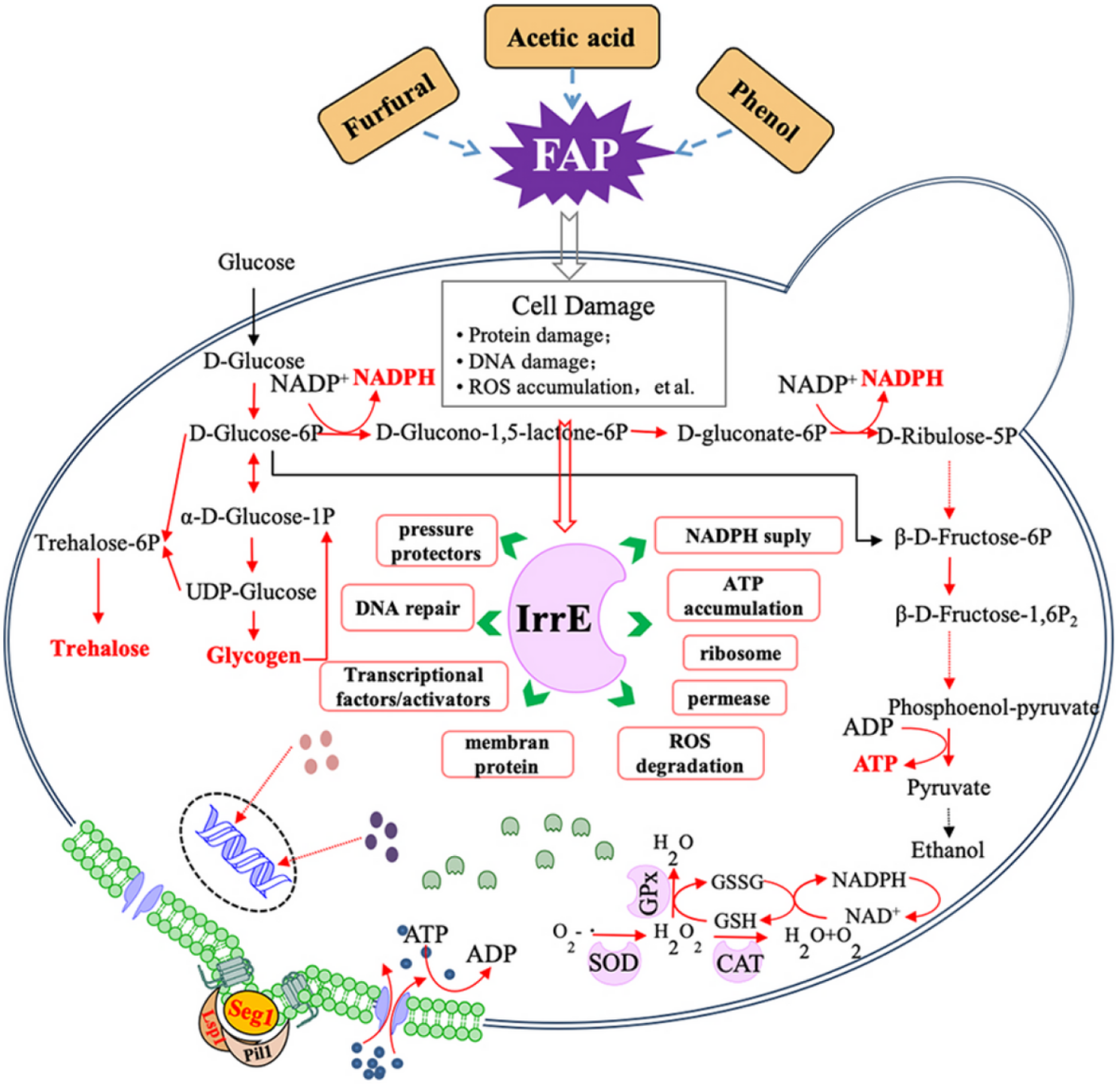

Figure 7

Hypothesis on the mechanisms of IrrE on the enhancement of tolerance to FAP.

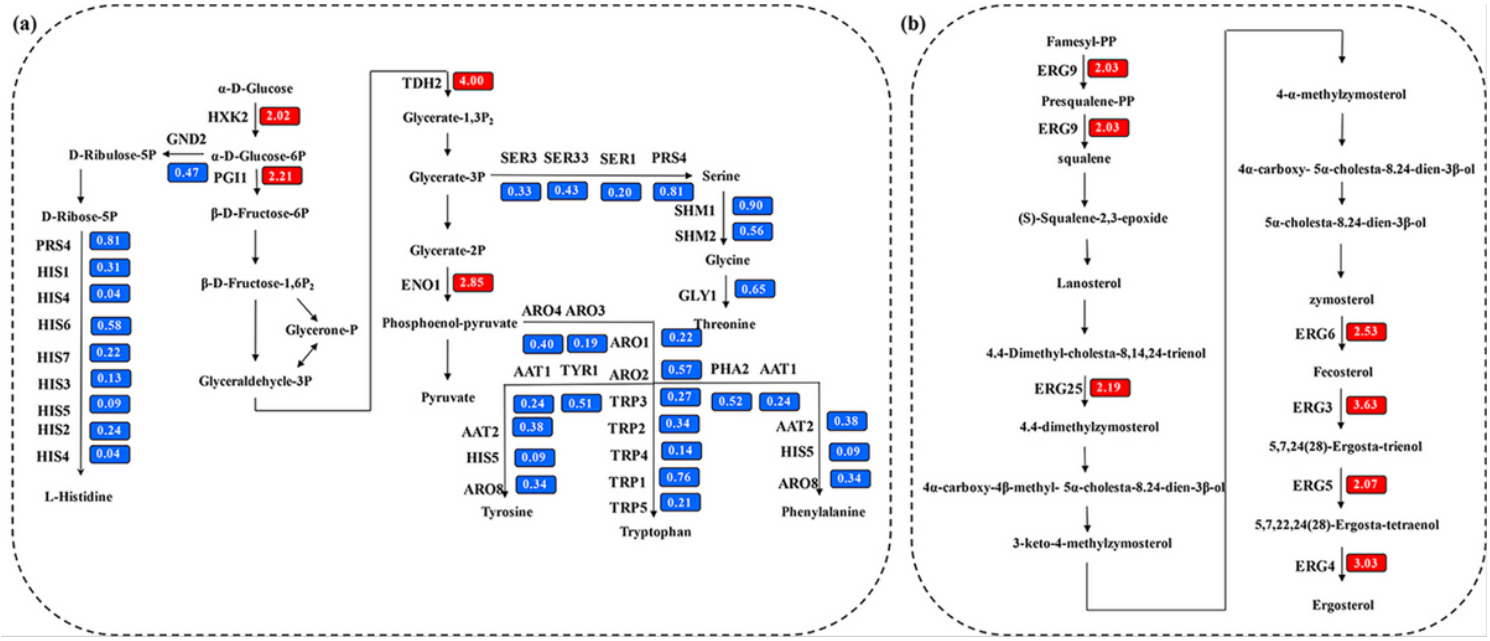

Figure 8

Effect of I24 on yeast transcription. Transcriptional profiles of energy metabolism (a) and ergosterol biosynthesis (b) by expressing I24. The strain BY4742/I24 and control strain BY4742/pRS416 were cultured in SC-Ura medium without FAP tolerance. Samples were taken in the middle of the lag phase. Box number exhibits transcriptional change, which is the foldchange of the transcriptional level of the strain BY4742/IrrE to that of the control strain BY4742/pRS416. Upregulated genes were highlighted in red.

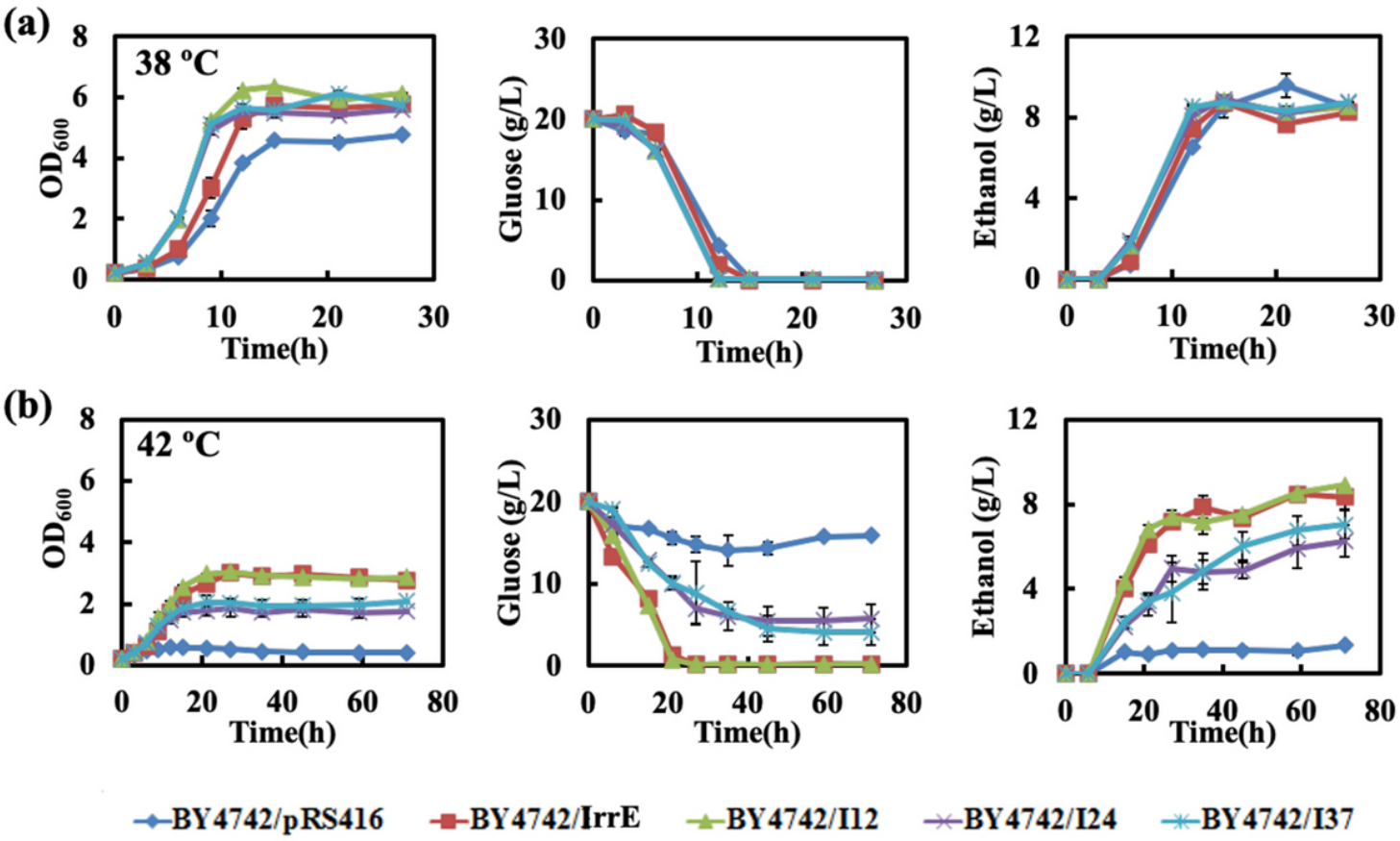

Figure 9

The effect of IrrE mutants on thermal tolerance. The strains were cultivated in SC-Ura medium at (a) 38 ºC and (b) 42 ºC. Results are the mean of duplicate experiments and error bars indicate s.d.

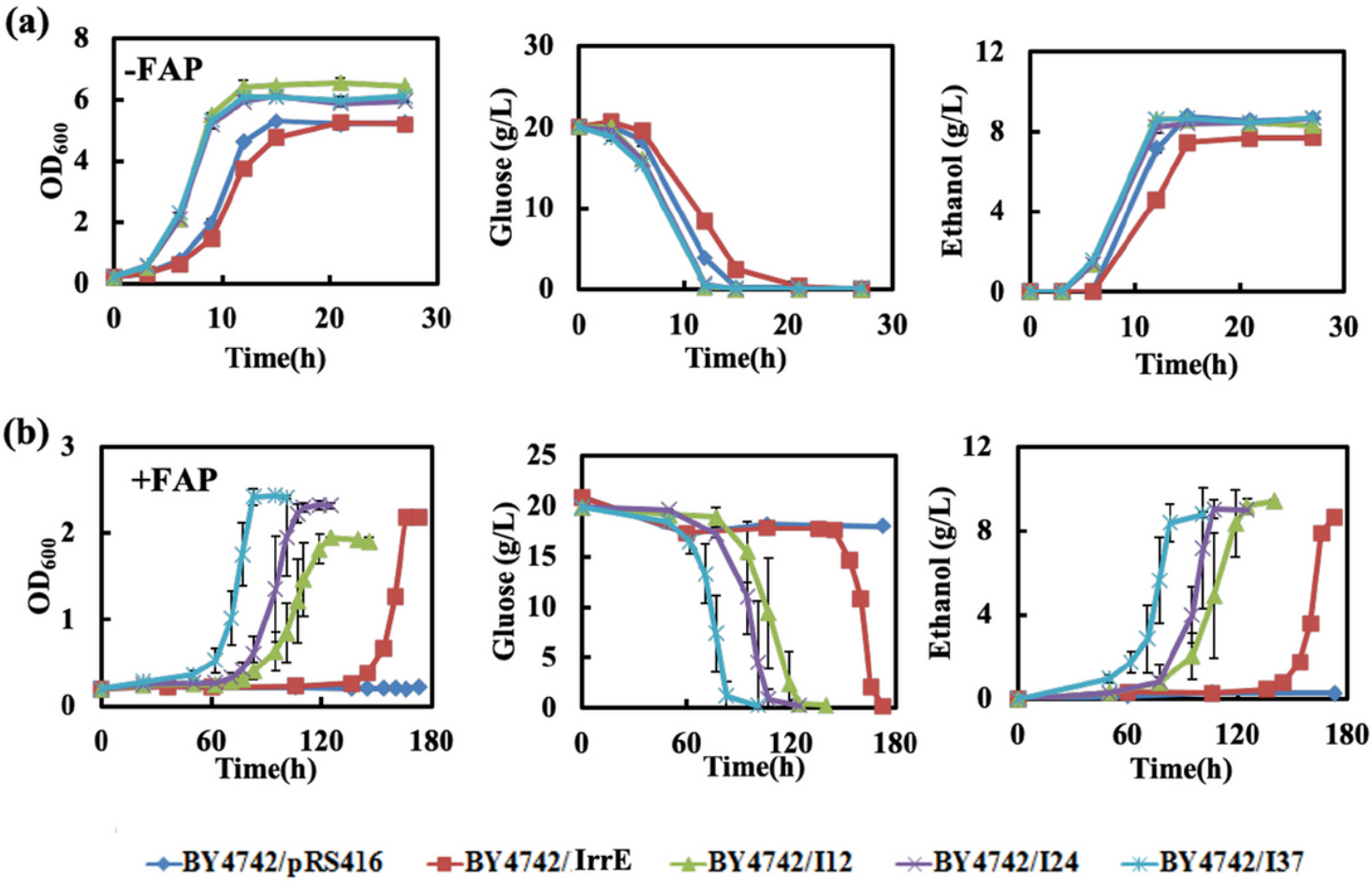

Figure 10

The fermentation profiles of the strain with IrrE mutants under FAP and thermal stress. The growth behaviors, glucose consumption and ethanol production of the strains at 34 ºC in the absence (a) and presence (b) of 0.8 g/L furfural, 3.0 g/L acetic acid and 0.3 g/L phenol. Results are the mean of duplicate experiments and error bars indicate s.d.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile2.docx
- Additionalfile1TableS1.xlsx