Enhancing *Saccharomyces cerevisiae* reactive oxygen species and ethanol stress tolerance for high-level production of protopanoxadiol

Fanglong Zhao, Yanhui Du, Peng Bai, Jingjing Liu, Wenyu Lu, Yingjin Yuan

PII: S0960-8524(16)31731-X
DOI: http://dx.doi.org/10.1016/j.biortech.2016.12.061
Reference: BITE 17437

To appear in: *Bioresource Technology*

Received Date: 4 October 2016
Revised Date: 16 December 2016
Accepted Date: 17 December 2016

Please cite this article as: Zhao, F., Du, Y., Bai, P., Liu, J., Lu, W., Yuan, Y., Enhancing *Saccharomyces cerevisiae* reactive oxygen species and ethanol stress tolerance for high-level production of protopanoxadiol, *Bioresource Technology* (2016), doi: http://dx.doi.org/10.1016/j.biortech.2016.12.061

Enhancing *Saccharomyces cerevisiae* reactive oxygen species and ethanol stress tolerance for high-level production of protopanoxadiol

Fanglong Zhao[a,1], Yanhui Du[a,1], Peng Bai[a,b], Jingjing Liu [a], Wenyu Lu[a,b,c]*, Yingjin Yuan[a,b,c]

[a] School of Chemical Engineering and Technology, Tianjin University, Tianjin 300350, PR China;

[b] Key Laboratory of System Bioengineering (Tianjin University), Ministry of Education, Tianjin, 300350, PR China;

[c] SynBio Research Platform, Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin, 300350, PR China.

* Correspondence to: School of Chemical Engineering and Technology, Tianjin University, Tianjin 300350, PR China. Tel: +86-22-85356523, Fax: +86-22-27400973.

E-mail address: wenyulu@tju.edu.cn (W. Lu)

[1]These authors contributed equally to this work.

Abstract:

Protopanaxadiol (PPD) is an active compound in *Panax ginseng*. Recently, an optimized PPD synthesis pathway contained a ROS releasing step (a P450-type PPD synthase, PPDS) was introduced into *Saccharomyces cerevisiae*. Here reported a synergistic effect of PPDS-CPR (CPR, cytochrome P450 reductase) uncoupling and ethanol stress on ROS releasing, which reduced cells viability. To build a robust strain, a cell wall integrity associated gene *SSD1* was high-expressed to improve ethanol tolerance, and ROS level decreased for 24.7%. Then, regulating the expression of an oxidative stress regulation gene *YBP1* decreased 75.2% of ROS releasing, and improved cells viability from 71.3±1.3% to 88.3±1.4% at 84 h. Increased cells viability enables yeast to produce more PPD through feeding additional ethanol. In 5 L fermenter, PPD production of W3a-ssPy reached to 4.25±0.18 g/L (19.48±0.28 mg/L/OD600), which is the highest yield reported so far. This work makes the industrial production of PPD possible by microbial fermentation.

Keywords：*Saccharomyces cerevisiae*, Cells viability, ROS tolerance, Ethanol stress tolerance, Protopanaxadiol

1. Introduction

Protopanaxadiol (PPD), a dammarane-type triterpene compound, is a promising antineoplastic and antidepressant candidate medicine (Xu et al., 2010). Since traditional extraction from *Panax ginseng* is time-consuming and labor-intensive, *de novo* synthesis pathway of PPD has been engineered in *S. cerevisiae* to produce PPD (Dai et al., 2013; Zhao et al., 2016). In previous study, the last step for hydroxylation of dammarenediol-II (DMD), catalyzed by a cytochrome P450-type PPD synthase (PPDS, CYP716A47) and its reductase CPR (cytochrome P450 reductase), releases a kind of special toxic compounds, reactive oxygen species (ROS) (Zhao et al., 2016). ROS are generated in cells as metabolic by-product, which include hydrogen peroxide, superoxide anion, and hydroxyl radical (Winterbourn, 2008). When accumulated to a certain level, this kind of compounds is known to damage DNA, proteins, lipids, cytoskeleton and cause programmed cell death (Jacobson, 1996). Beside cytochrome P450 monooxygenase, ROS can also be generated in a wide variety of enzymatic reactions catalyzed by mitochondrial respiratory chain enzymes, nitric oxide synthase, NADPH oxidase, and lipoxygenase (Giorgio et al., 2007).

In fermentation process, yeast cells are subject to the influence of specific environmental stress, which also results in ROS production (Chokshi et al., 2015; Kai et al., 2015). During fermentation of *S. cerevisiae*, sugar in broth was first metabolized to ethanol, which then further consumed as secondary carbon source. On this account, high level ethanol is one common stress on yeast growth during fermentation (Stanley et al., 2010a). Ethanol stress has effects on many different cellular behaviors related to changes

in membrane fluidity, protein misfolding and chromatin condensation (Ma & Liu, 2010). Therein, mitochondrial membrane is one of the main targets of ethanol, which is known to cause the uncoupling of the electron transport chain from the ATPase as well as to increase ROS production (Bailey et al., 1999).

Since ROS toxicity could reduce the fermentation performance and complicate the fermentation process (Mendes-Ferreira et al., 2010), researchers have managed to protect yeast cells against ROS toxicity. The central theme of responses to ROS is the transcriptional reprogramming of cells to express specific antioxidants (D'Autréaux & Toledano, 2007). In *S. cerevisiae*, transcription factor YAP1 is a main determinant of ROS tolerance whose function needs a YAP1-binding protein YBP1 (Gulshan et al., 2011). It was reported that expression of *YBP1* is a rate-limiting step for YAP1 binding during oxidative stress (Gulshan et al., 2011), so regulating the expression of *YBP1* may contribute to ROS elimination in production strain. For ethanol tolerance, cell wall is the first line to defense the external ethanol (Hara et al., 1976), and researches have reported that up-regulating genes related to cell wall structure improved ethanol tolerance (Ogawa et al., 2000; Chandler et al., 2004). ROS induced by ethanol was mainly due to the influence of high level ethanol on mitochondrial iron–sulfur cluster assembly system (Perez-Gallardo et al., 2013), so constructing integrated cell wall can realize physical separation between endomembrane system and extracellular ethanol.

S. cerevisiae are resistant to certain level of ethanol and ROS, but there are differences between commercial yeast strains in their resistance to stress conditions (Carrasco & Querol, 2001). Previously, *S. cerevisiae* W303-1a was used for production of

PPD (Zhao et al., 2016), however, this host is sensitive to ethanol and ROS (Stanley et al., 2010b). In this study, a synergistic effect of PPDS-CPR uncoupling and ethanol stress on ROS releasing was spotted. During batch fermentation, ROS released by PPDS and ATR1 uncoupling made yeast more sensitive to ethanol. This synergistic effect of different incentives on ROS release in metabolic engineering has been rarely reported. The dual stress conditions affected the cells metabolism, decreased cells viability. To bypass the limitation on PPD production, a strain improvement strategy was proposed: 1) constructing integrated cell wall to enhance ethanol tolerance and thus reduce ROS production; 2) improving expression of oxidative stress response regulator *YBP1* to consume intracellular ROS. For ethanol tolerance, several high expressing cell wall-related genes *HSP150* (Kapteyn et al., 1999), *SPI1* (Simões et al., 2006), *SSD1* (Kaeberlein & Guarente, 2002), *SED1* (Shimoi et al., 1998) and *TIP1* (Fujii et al., 1999) were tested respectively for enhancing ethanol tolerance. Gene *SSD1* was selected and highly expressed to improve ethanol tolerance. Then the expression of *YBP1* was regulated to consume intracellular ROS. After that, ROS level decreased 75.2% and cells viability improved from 73.6±1.4% to 86.3±1.3% at 84 h. Based on that, development of carbon source stage-controlled fermentation processes for the reengineered strain led to PPD production higher than 4 g/L. These results suggested that the growth stresses and their interactions during fermentation should be well regulated in engineering a robust industrial strain.

2. **Materials and Methods**

2.1 Strains and Media

Strain W2 and W3a was reported recently (Zhao et al., 2016) and stored at -80℃ in 25% glycerol. Genome of *S. cerevisiae* BY4741 was used for PCR amplification of *SSD1* and *YBP1* genes. *S. cerevisiae* strains grew in SD medium (Adams et al., 1998) lacking adenine, uracil, and histidine where appropriate.

2.2 Intracellular ROS level determination

S. cerevisiae cells from 1 mL of culture broth were collected by centrifugation at 4,000×g for 1 min. The cells were washed three times with 50 mM phosphate-buffered saline (PBS, pH 7.0, 4 °C) and then resuspended in 1 mL PBS. ROS levels were monitored with 2', 7'-dichlorofluorescein diacetate (Liu et al., 2013). Briefly, 1 mL resuspended cells was loaded with 1 μL of 10 mM DCFH-DA, and then incubated at 37 °C for 20min. Then cells were washed twice with PBS, and the DCFH fluorescence intensity was quantified using an F-4500 spectrophotometer (Hitachi, Japan) with an excitation wavelength of 488 nm and an emission filters of 525 nm (slit width, 10 nm). Values of the bars indicated the fold changes of ROS levels over control sample W2 at 24h.

2.3 Selecting genes to enhance ethanol tolerance and strain growth in plates, liquid dose–response analysis

In strain W3a, *URA3* and *HIS3* have been used as selection marker genes flanked by two loxP sites (Zhao et al., 2016). In this study, these two marker genes were firstly deleted using a Cre-expressing plasmid pSH63 (Gueldener, 2002), resulting in strain W3a-HU. Genes *HSP150* (Kapteyn et al., 1999), *SPI1* (Simões et al., 2006), *SSD1* (Kaeberlein & Guarente, 2002), *SED1* (Shimoi et al., 1998) *and TIP1* (Fujii et al., 1999)

that associated with cell wall integrity were integrated in *his3* site of W3a-HU. The resulted strains were listed in Table 1. Gene *SSD1* was integrated in *his3* site of W3a-HU, and resulted in strain W3a-ss.

2.4 Regulating expression of *YBP1* in strain W3a

Gene *YBP1* as well as its native promoter and terminator *YBP1p-YBP1-YBP1t* was amplified from genome of *S. cerevisiae* BY4741. Gene *YBP1*, promoters *PGK1p* and *TEF1p*, terminators *CYC1t* were amplified from genome of *S. cerevisiae* BY4741. *PGK1p-YBP1-CYC1t* and *TEF1p-YBP1-CYC1t* expression cassettes were constructed by fusion PCR. *YPB1p*, *PGK1p* and *TEF1p* promoted cassettes were integrated in *ura3* site of W3a-HU, obtaining strain W3a-y, W3a-Py and W3a-Ty (Table 1), respectively. *YPB1*, *PGK1* promoted cassette was integrated in *ura3* site of W3a-ss, obtaining strain W3a-ssPy.

2.5 Cells viability assay

Cell viability was determined by the methylene blue staining technique as Zhou described (Zhou et al., 2010). Briefly, yeast cells collected at 24 h, 60 h, 84 h, 120 h and 144 h were diluted to reach an OD of 0.5-0.6 at 600 nm. Then 100 μL of methylene blue was mixed with 100 μL of cells suspension. The number of stained (inactive) and unstained (active) cells were counted.

2.6 Spot assay of ethanol and ROS tolerance of the engineered yeast strains

The engineered yeast strains were cultured in YPD liquid medium for 12 h. Then 10-fold serial dilutions of the cultures were made. An aliquot (5μL) of each dilution were spotted onto plates containing YPD with 9% (v/v) ethanol or 0.05 μM H_2O_2 and cultured

at 30 °C for 3 days until colonies developed.

2.7 Cell wall integrity assay

Cell wall integrity assay was based on previous study (Ovalle et al., 1998). Briefly, yeast were grown overnight in YPD medium at 30 ℃. Cells were harvest by centrifugation (5,000g, 2 min) and washed three times with deionized water. Then the cells were re-suspended at OD600 = 0.5 in TE buffer (Tris/HCl, 50 mM, EDTA, 5 mM, at pH 7.5). Zymolyase 20 T (Zymo Research, USA) was added to the cells suspension with a final concentration of 20 μg/ml. Cell suspensions were incubated at 30 °C, and absorbance was determined at 600 nm every 5 min. The samples were vortexed before each determination. The degradation rate (DR) was defined as the absolute value of the slope of least-squares fit line.

2.8 Oxidative stress response genes *TRX2*, *SOD1*, *GSH1* and *GLR1* expression analysis

Strain W3a, W3a-ss, W3a-Py and W3a-ssPy were cultured as described in section 2.9.1 (glucose feed strategy). At 84 h, 5 mL yeast cells were mixed with 2 ml RNA protect reagent (Tiange, China) to preserve RNA integrity, and then they were stored in liquid nitrogen. Approximately 1×10^7 cells were used for the total RNA extraction using the RNeasy minikit (Tiangen, China). DNase I was used to remove DNA contamination. RNA concentration was measured with the NanoDrop ND 1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). 500 ng RNA was converted to cDNA using Maxima H Minus First Strand cDNA Synthe-sis Kit (Fermentas, USA).

Primers used for qPCR experiment were designed using oligo 7 and were listed in

Table 2. Gene *act1* was selected as internal reference gene (Pfaffl, 2001). qPCR was carried out on the LightCycler 480 II using ChamQ^{TM} SYBR® qPCR Master Mix (Vazyme, China). The reaction volume was 20 µl containing 10 µl 2× ChamQ SYBR qPCR Master Mix, 0.4 µl each primer, 0.4µl 50×ROX Reference Dye 1, 1 µl cDNA and 7.8 µl RNase-free ddH_2O. Thermal cycling conditions were set as follows: initial denaturation, 1 cycle of 95 ℃ for 10 min; amplification, 40 cycles of 95 ℃ for 10 s, 56 ℃ for 10 s and 72 ℃ for 10 s. C_T values of the target genes (*TRX2*, *SOD1*, *GSH1* and *GLR1*) were normalized to reference gene *act1*. The dates were presented as ratios of gene expression between the target gene and the reference gene *act1*. All assays were performed in triplicate, and the reaction without reverse transcriptase was used as a negative control to check the residual contaminating genomic DNA.

Table 2

2.9 Yeast cultivation and fermentation process

2.9.1 Flask fermentation

In flask fermentation, all strains were inoculated into 250 mL flask containing 30 mL YPD medium at 30 ℃ and 220 rpm.

For glucose feed strategy, at 48 h, 5 mL fed solution (pH 5.5) containing 500 g/L glucose, 9 g/L KH_2PO_4, 5.12 g/L $MgSO_4$•$7H_2O$, 3.5 g/L K_2SO_4, 0.28 g/L Na_2SO_4, 0.5 g/L adenine, 0.6 g/L uracil, 1.2 g/L lysine, 10mL/L trace elements solution and 12 mL/L vitamin solution were fed to the flask. The components of trace elements solution and vitamin solution were described previously (Lenihan et al., 2008).

For restricted glucose feed strategy, 0.6 mL fed solution mentioned above was added

to the medium repeatedly at 24 h, 30 h, 38 h, 54 h, 62 h, 72 h, 88 h, 110 h, and 128 h, respectively, and the ethanol concentration could be controlled at a range of 1-6 g/L.

For extended fermentation process, 5 mL fed solution was added into medium at 48h. From 110 h, glucose solution or ethanol was further added into medium. Particularly, for glucose feeding, 0.5 mL glucose (100 g/L) solution was added repeatedly at 110 h, 120 h, 130 h, 144 h, 156 h and 168 h. For ethanol feeding, 200 μL ethanol (95%, v/v) was added to the medium repeatedly at 110 h, 116 h, 128 h, 144 h, 168 h and 186 h. The ethanol concentration could be controlled at a range of 1-6 g/L after 110 h.

2.9.2 Carbon source stage-controlled fermentation in 5 L bioreactor

Strains W3a-ssPy was fermented in 5 L bioreactors. 2 L YPD medium was added into 5 L fermenter (BLBIO-5GL, ShangHai Bailun Bio-Technology CO., LTD, China). To reduce the fermentation period, inoculum proportion was improved to 15%. 300 mL seed solution (incubated at 30 ℃ for 18 h) was inoculated into 2 L YPD medium. Fermentation was carried out at 30 ℃ with an DO higher than 40% and air flow rate higher than 2 L/min•L. The pH was controlled at 5.5 by automatic addition of 5 M ammonia hydroxide. At 26 h, 350 mL fed solution as mentioned in part 2.9.1 was fed to the bioreactors. Ethanol (95% v/v) was added from 84 h and the concentration of ethanol was maintained at 1-6 g/L.

2.10 Quantification of glucose and ethanol

Glucose and ethanol were measured with a semi-automatic bioanalyzer (SBA-40C, Shandong academy of sciences, China) according to the manufacturer's instructions. If concentrations were above the maximum assay range, samples were diluted with PBS

buffer.

2.11 OD600 measurement

During fermentation, some PPD was secreted into extracellular environment. Since PPD is poorly soluble in water, PPD precipitation will generate. The precipitation can be dissolved in ethyl acetate. So, the precipitation was extracted using ethyl acetate before OD600 measurement. 2 mL fermentation broth was mixed with 8 mL ethyl acetate, and the mixture was agitated by vortex for 5 min. Then the mixture was centrifuged at 12,000g for 5 min, and the liquid phase was discarded. The cells were resuspended in 2 mL deionized water. OD600 of the diluted aliquots was measured using a UV-Vis spectrophotometer (Oppler, 752 N, China). Deionized water was used as blank control.

2.12 PPD extraction and analysis

The PPD present in the intracellular and extracellular space during shake flask experiments. For extracellular PPD, 2 mL fermentation broth was mixed with 8 mL ethyl acetate, and the mixture was agitated by vortex for 5 min. Then the mixture was centrifuged at 12,000g for 5 min, and the ethyl acetate phase was collected. Intracellular PPD was extracted as follow: the supernatant was discarded and the cells were disrupted by vortex agitation with 0.5 mL ethyl acetate and 0.25 mL glass beads (0.5 mm diameter) for 10 min, followed by ultrasonic extraction. Intracellular PPD extraction process was repeated for 4 times, the ethyl acetate phase was combined for HPLC analyzing.

For 5 L fermentation, most of PPD was secreted out of cells and attached on inner tank wall and the surface of stainless pipe. After fermentation, solid substance was collected and dissolved in ethyl acetate. PPD in cells and the precipitation in medium

were extracted as described above.

HPLC analysis was carried on an Elite P230II high-pressure pump system equipped with UV detection at 203 nm. Chromatographic separation was realized on Hypersil C18 column (4.6 mm × 250 mm, 5 μm; Elite Analytical Instruments Co., Ltd., Dalian, China) at 30 ℃. Methanol–acetonitrile (4:6, v/v) was used as mobile phase.

3. Results and discussion

3.1 Synergistic effect of PPDS-CPR uncoupling and ethanol stress on ROS releasing

Recently, a PPD producing yeast strain which generated ROS by the uncoupling of PPDS and its reductase ATR1 was reported (Zhao et al., 2016). ROS accumulated gradually until 60 h during batch fermentation in shake-flask (Fig.1C). The intracellular ROS level of W3a was increased dramatically at 84 h, which was 11-fold higher than the reference strain W2 at 24 h (Fig.1C). Meanwhile, cell metabolism of W3a was affected. For strainW2 fermentation, the glucose was depleted at 86 h, while for strain W3a, the glucose depleted time was delayed for 4 h at 90 h (Fig.1B).

Figure 1

During fermentation of W2 and W3a, ethanol concentration was 25.1 and 24.3 g/L at 84 h, respectively. In consideration of the sensitive of the parent strain W303-1a to ethanol (Stanley et al., 2010b), it was speculated that the sudden rise of ROS level may be related to high ethanol concentration in the medium, since high-level ethanol also stimulated the production of ROS (Stanley et al., 2010a). To test that hypothesis, cells of W3a and W2 growing for 48 h (ethanol was depleted) were incubated in the medium containing 8-30 g/L ethanol for 4 h. The results showed that when ethanol concentration exceeded 18 g/L, intracellular ROS level of W3a began to rise (Fig.1D). While

intracellular ROS level of W2 began to rise when ethanol concentration exceeded 22 g/L (Fig.1D). This implied that strain W3a was more sensitive to ethanol than strain W2. High ethanol concentration in the medium (exceeded 18 g/L for strain W3a) would accelerate ROS releasing. Otani et al. have found that expression of CYP2E1 (a P450 monooxygenase), which is a well-documented source of ROS, in hepatoma cells also resulted in increased sensitivity to ethanol and other oxidants (Otani et al., 2005). High level of ethanol exposure results in alterations in mitochondrion membrane fluidity, structure and function, which ultimately disrupts electron transport chain and results in generation of ROS (Bailey et al., 1999). Moreover, mitochondrion is also a target of ROS (Kim et al., 2003). Increased sensitivity to ethanol of W3a might be due to the fact that ROS released by PPDS impaired mitochondrion, and decreased the threshold of ethanol toxicity.

Based on these results, ethanol concentration should be controlled under 18 g/L during glucose-fed fermentation process. To this end, restricted glucose feed strategy was used in shaking flask fermentation by controlling ethanol concentration at a range of 1-6 g/L. But the yield of PPD was only 23.2 mg/L/OD600 (960.8 mg/L), which might be related to glucose repression (Carlson, 1999). Therefore, genetic engineering approach was harnessed to improve resistance against ethanol and ROS and increase strain robustness.

3.2 High-expression of *SSD1* and *YBP1* conferring interaction effect on improving cells viability

To construct a robust cell for PPD production, extracellular ethanol stress and

intracellular ROS should be treated simultaneously. The cell wall is the first line of defense against toxic effects of ethanol (Hara et al., 1976). For *S. cerevisiae*, the integrated cell wall could enhance ethanol stress by physical separation. In this study, gene *SSD1* was selected as candidate to improve ethanol tolerance from 5 genes (*HSP150*, *SPI1*, *SSD1*, *SED1* and *TIP1*) involving in cell-wall synthesis. The results demonstrated that expression of *SSD1* could improve ethanol tolerance with no negative effect on PPD synthesis (Fig.2E). In addition, high expression of *SSD1* decreased ROS level for 24.7% at 84h (Fig.2C). This result further indicated ethanol stress is one additional source of ROS at 84h.

Table 1 Figure 2

In *S. cerevisiae*, transcription factor YAP1 acts as a major regulator of oxidative stress response. Upon activation by high level of ROS, YAP1 rapidly redistributes to the nucleus where it regulates the expression of antioxidative genes (Wood et al., 2004). The activation of YAP1 nuclear localization requires the participation of a YAP1-binding protein YBP1 (Gulshan et al., 2011). Particularly, *S. cerevisiae* W303-1a have a mutant allele of *YBP1* (*ybp*1-1) and exhibit sensitivity to H_2O_2 (Veal et al., 2003). In this study, *YBP1* cassette as well as its native promoter was integrated in strain W3a. But the ROS tolerance of the resulted strain W3a-y was not improved. Expression of *YBP1* was further controlled under strong promoter *PGK1p* and *TEF1p* and then strain W3a-Py and W3a-Ty was achieved respectively. ROS tolerance of these two strains were improved, while when compared to their parent strain W3a, PPD production decreased by about 10% (Fig.2E). Theoretically, biosynthesis of 1 mol PPD from Acetyl-CoA needs 15 mol NADPH in *S.*

cerevisiae, meanwhile, detoxifying ROS also consume a large amount of NADPH (Godon et al., 1998). Thus cells need more reducing power to counterbalance intracellular ROS when subjected to oxidative stress and that this process may reduce NADPH supply for PPD synthesis.

Cells viability is of crucial importance for high production of PPD in fermentation process. High-expression of *SSD1* or/and *YBP1* could improve cells viability to different degrees (Fig.2D). It is noteworthy that the cells viability of strain W3a-ssPy was higher than that of strain W3a-ss and W3a-Py (Fig.2D). Oxidative stress is a side effect of ethanol stress. The integrated cells wall conferred by *SSD1* could enhance ethanol tolerance and reduce ROS generation (Fig.2C). In addition, consuming ROS could also decrease ethanol toxicity (Du & Takagi, 2007). So high-expression of *YBP1* also contributed to improve ethanol tolerance.

3.3 Cell wall integrity assay and oxidative stress response gene expression analysis

To further verify the function of high expression of *SSD1* gene on improving cells wall integration, cell wall integrity assay was performed using zymolyase digestion method (Ovalle et al., 1998). The OD600 of the yeast cells suspension decreases as the cells lyse, and the time course is thought to depend on thickness of the cell wall (Jung et al., 2005). The degradation rate (DR) of strains W3a, W3a-Py, W3a-ss and W3a-ssPy were 0.23±0.03 h^{-1}, 0.25±0.02 h^{-1}, 0.18±0.02 h^{-1} and 0.17±0.03 h^{-1}, respectively. The result indicated that the cell walls of strains W3a-ss and W3a-ssPy were more stable than that of strains W3a and W3a-Py.

Figure 3

For strain W3a-Py and W3a-ssPy, H_2O_2 tolerance was enhanced (Fig.2B). This result indicated that YBP1 was worked in these strains. Thioredoxin, glutathione and superoxide dismutase (SOD) are well known antioxidant systems to protect their cellular constituents and maintain cellular redox state (Finkel, 2003). In *S. cerevisiae*, expression of *TRX2* (thioredoxin), *SOD1* (superoxide dismutase), *GSH1* (glutamate-cysteine ligase), and *GLR1* (glutathione reductase) are up-regulated by YAP1 under oxidation conditions (Jamieson, 1998). Since YAP1-binding protein YBP1 is scarce in parent strain W303-1a, high expression of *YBP1* in W3a-Py and W3a-ssPy may increase *TRX2*, *SOD1*, *GSH1* and *GLR1* expression level under oxidative stress. Here, expressions of these genes at 84 h in engineered strain were analyzed. The result in Fig.3B showed that the expression of *TRX2*, *SOD1*, *GSH1*, and *GLR1* increased up to 1.8-8 folds after *YBP1* was overexpressed. This result may account for the decrease of ROS level in strain W3a-Py and W3a-ssPy.

3.4 PPD productions of W3a and W3a-ssPy during fermentation in flask.

In glucose-fed fermentation, PPD titer by strain W3a-ssPy decreased 12.6±0.53% (from 1435.7±41.6 to 1254.7±56.3 mg/L) compared to parent strain W3a (Fig.4A). The maximum rate of ethanol consumption and PPD synthesis by strain W3a-ssPy were both higher than strain W3a. For example, from 60 to 120 h, maximum PPD synthesis rate (μ_{PPD}) of W3a-ssPy was 15.85±0.32 mg/(h·L), which was higher than that of W3a (13.96±0.23 mg/(h·L)). From 84 to 120 h, maximum ethanol consumption rate ($-\mu_{ethanol}$) of strain W3a-ssPy was 0.95±0.07 g/(h·L), while $-\mu_{ethanol}$ of strain W3a was 0.76±0.06 g/(h·L). Thus, the promotion of cells viability was helpful to increase synthetic efficiency of PPD.

Figure 4

In addition, Fig.4A and B showed that synthetic rate of PPD decreased rapidly with ethanol depleted. For W3a-ssPy, ethanol was depleted at 110 h. At the same time, strain W3a-ssPy maintained high cells viability (Fig.4D). To test whether further feeding additional carbon source contributed to improving PPD production, glucose solution or ethanol was added to the medium at 110 h. For strain W3a-ssPy, glucose feeding did not increase the production of PPD (Fig.4C), but controlling ethanol concentration at a range of 1-6 g/L after 110 h gave an increase in production of PPD, attaining 33.7±1.9 mg/L/OD600 (1806.4±34.5 mg/L) at 168 h (Fig.4C). Under the same conditions, productions of strain W3a were not improved, no matter what feeding carbon source were used (Fig.4C). In extended fermentation process, strain W3a-ssPy grew more robust than W3a (Fig.4D), which might account for the production enhancement of W3a-ssPy.

3.5 Production of PPD in 5 L bioreactor based on carbon source stage-controlled fermentation

Glucose and ethanol are commonly used carbon sources for yeast production of terpenoids compounds. Previously, 41 g/L of amorpha-4,11-diene (precursor of artemisinin) has been achieved with the use of ethanol feed (Westfall et al., 2012). In this study, the flask fermentation experiment demonstrated that ethanol was more favorable than glucose as additional feeding carbon source on PPD production. However, ethanol is more expensive than glucose on a weight basis. In addition, when growing on ethanol, the rate of yeast biomass production is lower than that on glucose (Zampar et al., 2012). To develop a cost-effective production process, 5 L fermentation was carried out based on carbon source stage-controlled fermentation (Fig.5A). On stage 1, glucose was fed for

rapid growth. At the end of stage 1, glucose was depleted and then, on stage 2, additional ethanol was fed into medium and dominated as the sole carbon source for PPD production.

PPD existed in yeast cells and PPD precipitation in medium during shake flask fermentation. While in 5 L fermenter, PPD was mainly secreted to extracellular space and adhered to the surface of stainless pipe and the inner tank wall as faint yellow solid. This feature made it impossible to determine PPD production in real time during 5 L fermentation. So 3 batches (batch NO 1, NO 2, and NO 3) fermentation terminated at 120 h, 144 h and 168h respectively were performed to determine the end time. Finally, PPD productions of the batch NO 1, NO 2, and NO 3 were 18.99 mg/L/OD600, 19.72 mg/L/OD600 and 18.87 mg/L/OD600, respectively (Table 3). These results indicated that fermentation could be stopped at 144 h.

Table 3 Figure 5

To further verify the reliability, this fermentation process was conducted for another two batches (named batch NO 4 and NO 5). Both of them were terminated at 144 h. Fig. 5 showed the average date of batch NO 2, NO 4, and NO 5. On stage 1, glucose feeding was started at 26 h when initial glucose and its metabolite ethanol were exhausted (Fig.5A). After 26 h, the feed glucose was metabolized into ethanol, and ethanol concentration reached maximum at 60 h, attaining about 26 g/L (Fig.5A). At 84 h, ethanol concentration decreased under 1 g/L, and then stage 2 began when additional ethanol was fed to the fermenter and maintained concentration at a range of 1-6 g/L. OD600 kept increasing by 120 h, reaching to 218.4±4.5 (Fig.5A).

In this study, PPD production reached 4 g/L, which were three- to fourfolds higher than Dai (Dai et al., 2013) and Zhao (Zhao et al., 2016) reported. Mosesa et al. reported that efficiently transferring triterpenes from yeast cells into the culture medium could dramatically increase saponins production levels (Moses et.al, 2014). Dai et al. developed two-phase extractive fermentation for enhancing DMD and PPD productions (Dai et al., 2013). However, recent study found that two-phase extractive fermentation also accelerated DMD secretion, preventing conversion of DMD (Zhao et al., 2016). Since high-efficient catalytic conversion of DMD is crucial for high production of PPD, two-phase extractive fermentation is not proper. In 5L fermentation, about 98% of PPD can pass the membrane even without addition of organic solvent (Fig.5B). On this occasion, conversion ratio of DMD was kept beyond 96% (Table 3).

4. Conclusion

In summary, the synergistic effect of P450-CPR uncoupling and ethanol stress on ROS releasing was found in this study. During fed fermentation, high level ROS influenced cells vitality and resulted in lower PPD productivity. To bypass this limitation, a strain improvement strategy that enhancing cell wall integrity to resist extracellular ethanol and increasing oxidative stress response to consume intracellular ROS was proposed. Results showed that this strategy was effective to keep cells robust when genes *SSD1* and *YBP1* were both high expressed in a ROS and ethanol sensitive strain. As a result, PPD production in W3a-ssPy reached to 4.25 ± 0.18 g/L in 5 L fermenter, which is the highest PPD production ever reported using *S. cerevisiae*.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors acknowledge the financial support provided by the National Basic Research Program of China ("973" Program: 2012CB721105), the Ministry of Science and Technology of China ("863"Program: 2012AA02A701) and National Natural Science Funds for Innovation Research Groups 21621004.

References

1. Adams, A., Gottschling, D., Kaiser, C., Stearns, T., 1998. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
2. Allen, S.A., Clark, W., McCaffery, J.M., Cai, Z., Lanctot, A., Slininger, P.J., Liu, Z.L., Gorsich, S.W., 2010. Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. Biotechnol. Biofuels. **3**, 2.
3. Bailey, S.M., Pietsch, E.C., Cunningham, C.C., 1999. Ethanol stimulates the production of reactive oxygen species at mitochondrial complexes I and III. Free. Radic. Biol. Med. **27**, 891-900.
4. Carlson, M., 1999. Glucose repression in yeast. Curt. Opin. Microbiol. **2**, 202-207.
5. Carrasco, P., Querol, A., 2001. Analysis of the stress resistance of commercial wine yeast strains. Arch. Microbiol. 175, 450-457.
6. Chandler, M., Stanley, G.A., Rogers, P., Chambers, P., 2004. A genomic approach to defining the ethanol stress response in the yeast *Saccharomyces cerevisiae*. Ann Microbiol. **54**:427–454.
7. Chokshi, K., Pancha, I., Trivedi, K., George, B., Maurya, R., Ghosh, A., Mishra, S., 2015. Biofuel potential of the newly isolated microalgae acutodesmus dimorphus, under temperature induced oxidative stress conditions. Bioresour. Technol. **180**, 162-171.
8. D'Autréaux, B., Toledano, M.B., 2007. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat. Rev. Mol. Cell. Bio. **8**, 813-824.
9. Dai, Z., Liu, Y., Zhang, X., Shi, M., Wang, B., Wang, D., Huang, L., Zhang, X., 2013. Metabolic engineering of *Saccharomyces cerevisiae* for production of ginsenosides. Metab. Eng. **20**, 146-156.
10. Daum, G., Lees, N.D., Bard, M., Dickson, R.,1998. Biochemistry, cell biology, and molecular biology of lipids of *Saccharomyces cerevisiae*. Yeast. **14**, 1471–1510
11. Du, X., Takagi, H., 2007. N-Acetyltransferase Mpr1 confers ethanol tolerance on *Saccharomyces cerevisiae* by reducing reactive oxygen species. Appl. Microbilo. Biotechnol. **75**, 1343-1351.
12. Finkel, T., 2003. Oxidant signals and oxidative stress. Curr. Opin. Cell. Biol. **15**, 247-254.
13. Fujii, T., Shimoi, H., Iimura, Y., 1999. Structure of the glucan-binding sugar chain of Tip1p, a cell wall protein of Saccharomyces cerevisiae. Biochim. Biophys. Acta. **1427**, 133–144.
14. Giorgio, M., Trinei, M., Migliaccio, E., Pelicci, P.G., 2007. Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? Nat. Rev. Mol. Cell. Bio. **8**, 722-728.
15. Godon, C., Lagniel, G., Lee, J., Buhler, J.-M., Kieffer, S., Perrot, M., Boucherie, H., Toledano, M.B., Labarre, J., 1998. The H_2O_2 stimulon in *Saccharomyces cerevisiae*. J. Biol. Chem. **273**, 22480-22489.

16. Gueldener, U., Heinisch, J., Koehler, G. J., Voss, D., Hegemann, J. H., 2002. A second set of loxp marker cassettes for cre-mediated multiple gene knockouts in budding yeast. Nucleic. Acids. Res. **30**, 88-94.
17. Gulshan, K., Lee, S.S., Moye-Rowley, W.S., 2011. Differential oxidant tolerance determined by the key transcription factor Yap1 is controlled by levels of the Yap1-binding protein, Ybp1. J. Biol. Chem. **286**, 34071-34081.
18. Hara, S., Sasaki, M., Obata, T., Noshiro, K., 1976. Isolation of ethanol-tolerant mutants from sake yeast Kyokai no. 7. J. Brew. Soc. Jpn. **71**, 301-304.
19. Jacobson, M. D., 1996. Reactive oxygen species and programmed cell death. Trends. Biochem. Sci. **21**:83-86.
20. Jamieson, D. J., 1998. Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. Yeast. **14**, 1511-1527.
21. Jung, W. H., Warn, P., Ragni, E., Popolo, L., Nunn, C. D., Turner, M. P., Stateva, L., 2005. Deletion of *PDE2*, the gene encoding the high-affinity cAMP phosphodiesterase, results in changes of the cell wall and membrane in *Candida albicans*. Yeast. **22**, 285-294.
22. Kaeberlein, M., Guarente, L., 2002. *Saccharomyces cerevisiae* MPT5 and SSD1 function in parallel pathways to promote cell wall integrity. Genetics. **160**, 83-95.
23. Kai, Q., Xia, X.X., Zhong, J.J., 2015. Enhanced anti-oxidative activity and lignocellulosic ethanol production by biotin addition to medium in *Pichia guilliermondii* fermentation. Bioresour. Technol. **189**:36-43.
24. Kapteyn, J.C., Egmond, P.V., Sievi, E., Ende, H.V.D., Makarow, M., Klis, F.M., 1999. The contribution of the O -glycosylated protein Pir2p/Hsp150 to the construction of the yeast cell wall in wild-type cells and β1,6-glucan-deficient mutants. Mol. Microbiol. **31**, 1835-1844.
25. Kim, J., He, L., Lemasters, J., 2003. Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. Biochem. Biophys. Res. Commun **304**, 463-470.
26. Lenihan, J. R., Tsuruta, H., Diola, D., Renninger, N. S., Regentin, R., 2008. Developing an industrial artemisinic acid fermentation process to support the cost-effective production of antimalarial artemisinin-based combination therapies. Biotechnol. Progr. **24**, 1026-32.
27. Liu, J.D., Zhu, Y.B., Du, G.C., Zhou, J.W., Chen, J., 2013. Response of *Saccharomyces cerevisiae* to D-limonene-induced oxidative stress. Appl. Microbiol. Biotechnol. **97**, 6467-6475.
28. Ma, M., Liu, Z.L., 2010. Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **87**, 829-845.
29. Mendes-Ferreira, A., Sampaio-Marques, B., Barbosa, C., Rodrigues, F., Costa, V., Mendes-Faia, A., Ludovico, P., Leão, C., 2010. Accumulation of non-superoxide anion reactive oxygen species mediates nitrogen-limited alcoholic fermentation by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **76**, 7918-7924.
30. Moses, T., Pollier, J., Almagro, L., Buyst, D., Van, M. M., Pedreño, M. A., et al. 2014. Combinatorial biosynthesis of sapogenins and saponins in *Saccharomyces cerevisiae* using a c-16α hydroxylase from *Bupleurum falcatum*. PNAS, **111**, 1634-9.
31. Ogawa, Y., Nitta, A., Uchiyama, H., Imamura, T., Shiomoi, H., Ito, K., 2000. Tolerance mechanism of the ethanol-tolerant mutant of sake yeast. J. Biosci. Bioeng. **90**:313–320
32. Otani, K., Korenaga, M., Beard, M.R., Li, K., Qian, T., Showalter, L.A., Singh, A.K., Wang, T., Weinman, S.A., Martinsf, J.C., Theveleinc, J.M., Goossensa, A., 2005. Hepatitis C virus core protein, cytochrome P450 2E1, and alcohol produce combined mitochondrial injury and cytotoxicity in

hepatoma cells. Gastroenterology. **128**, 96-107.

33. Ovalle, R., Lim, S. T., Holder, B., Jue, C. K., Moore, C. W., Lipke, P. N., 1998. A spheroplast rate assay for determination of cell wall integrity in yeast. Yeast, **14**, 1159-1166.
34. Perez-Gallardo, R. V., Briones, L. S., Díaz-Pérez, A. L., Gutiérrez, S., Rodríguez-Zavala, J. S., & Campos-García, J., 2013. Reactive oxygen species production induced by ethanol in *Saccharomyces cerevisiae* increases because of a dysfunctional mitochondrial iron–sulfur cluster assembly system. FEMS. YEAST. RES. **13**, 804-819.
35. Pfaffl, MW., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic. Acids. Res. **29**:e45.
36. Shimoi, H., Kitagaki, H., Ohmori, H., Iimura, Y., Ito, K., 1998. Sed1p is a major cell wall protein of *Saccharomyces cerevisiae* in the stationary phase and is involved in lytic enzyme resistance. J. Bacteriol. **180**, 3381-7.
37. Simões, T., Mira, N.P., Fernandes, A.R., Sácorreia, I., 2006. The *SPI1* gene, encoding a glycosylphosphatidylinositol-anchored cell wall protein, plays a prominent role in the development of yeast resistance to lipophilic weak-acid food preservatives. Appl. Environ. Microbiol. **72**, 7168-7175.
38. Stanley, D., Bandara, A., Fraser, S., Chambers, P., Stanley, G.A., 2010a. The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. J. Appl. Microbiol. **109**, 13-24.
39. Stanley, D., Chambers, P. J., Stanley, G. A., Borneman, A., Fraser, S., 2010b. Transcriptional changes associated with ethanol tolerance in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **88**, 231-9.
40. Vanegas, J., Contreras, M., Faller, R., Longo, M., 2012. Role of unsaturated lipid and ergosterol in ethanol tolerance of model yeast biomembranes. Biophys. J. **102**, 507-16.
41. Veal, E.A., Ross, S.J., Malakasi, P., Peacock, E., Morgan, B.A., 2003. Ybp1 is required for the hydrogen peroxide-induced oxidation of the Yap1 transcription factor. J. Biol. Chem. **278**, 30896-30904.
42. Westfall, P. J., Pitera, D. J., Lenihan, J. R., Eng, D., Woolard, F. X., Regentin, R., Horninga, T., Tsurutaa, H., Melisa, D.J., Owensa, A., Fickesa, S., Diolaa, D., Benjamina, K.R., Keasling, J.D., Leavella, M.D., McPheea, D.J., Renningera, N.S., Newmana, J.D., Paddona, C.J., 2012. Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. PNAS. **109**, E111-E118.
43. Winterbourn, C.C., 2008. Reconciling the chemistry and biology of reactive oxygen species. Nat. Chem. Biol. **4**, 278-286.
44. Wood, M. J., Storz, G., Tjandra, N., 2004. Structural basis for redox regulation of Yap1 transcription factor localization. Nature, 430:917-21.
45. Xu, C.J., Teng, J.J., Chen, W.D., Ge, Q., Yang, Z.Q., Yu, C.Y., Yang, Z.R., Jia, W., 2010. 20 (S)-protopanaxadiol, an active ginseng metabolite, exhibits strong antidepressant-like effects in animal tests. Prog. Neuro-Psychoph. **34**, 1402-1411.
46. You, K.M., Rosenfield, C.L., Knipple, D.C., 2003 Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. Appl. Environ. Microbiol **69**, 1499–1503.
47. Zampar, G. G., Kümmel, A., Ewald, J., Jol, S., Niebel, B., Picotti, P., Aebersold, R., Sauer, U., Zamboni, N., Heinemann, M., 2012. Temporal system-level organization of the switch from glycolytic to gluconeogenic operation in yeast. Mol. Syst. Biol. **9**, 651-651.
48. Zhao, F.L., Bai, P., Liu, T., Li, D.S., Zhang, X.M., Lu, W.Y., Yuan, Y.J., 2016. Optimization of a cytochrome P450 oxidation system for enhancing protopanaxadiol production in *Saccharomyces*

cerevisiae. Biotechnol. Bioeng. **113**, 1787–1795.

49. Zhou, X., Zhou, J., Tian, H.C., Yuan. Y.J., 2010. Dynamic lipidomic insights into the adaptive responses of *Saccharomyces cerevisiae* to the repeated vacuum fermentation. OMICS. **14**, 563-574.

Figure Captions

Figure 1. Growth status, substrate consumption and intracellular ROS level of strains W2 and W3a during glucose-fed fermentation in flasks. (A) Growth curves of strains W2 and W3a. (B) Glucose and ethanol concentration in medium. (C) ROS levels of strains W2 and W3a in fermentation process. (D) ROS levels of strains W2 and W3a under different ethanol concentration for 4 h. Values of the bars indicated the fold changes of ROS levels over control sample W2 at 24h.

Figure 2. High-expressions of *SSD1* and *YBP1* conferring interaction effect on ethanol and ROS tolerance. High-expression of *SSD1* or/and *YBP1* on (9%, v/v) ethanol plate (A) and 0.05 uM H_2O_2 plate (B). ROS level (C) and cells viability (D) of engineered strains in fed fermentation process. Values of the bars indicated the fold changes of ROS levels over control sample W2 at 24h. (E) PPD production of engineered strains in YPD medium after 4 days culture.

Figure 3. Cell wall integrity assay and oxidative stress response gene expression analysis. (A) Time course of OD600 during zymolyase digestion. The degradation rate (DR) was defined as the absolute value of the slope of least-squares fit line. (B) qPCR studies to determine expression of *TRX2*, *SOD1*, *GSH1* and *GLR1* in strain W3a, W3a-ss,W3a-Py, and W3a-ssPy. Data represented the relative abundances of target genes in each strain with respect to that of housekeeping gene *act1*.

Figure 4. Comparisons of PPD production (A), and ethanol consumption (B) between strains W3a and W3a-ssPy. Dotted lines indicated the linear fitting of the dates. PPD

production of strains after extended fermentation in flask (C). From 110 h, glucose (100 g/L) solution or ethanol (95%) was added repeatedly to the medium, and the ethanol concentration was controlled at a range of 1-6 g/L. (D) Cells viability during extended fermentation in flask.

Figure 5. Results of 5 L fermentation. (A) OD600, glucose and ethanol consumption during fermentation in 5L bioreactor. (B) PPD proportion in faint yellow solid, PPD precipitation in medium and cells.

Figure 1

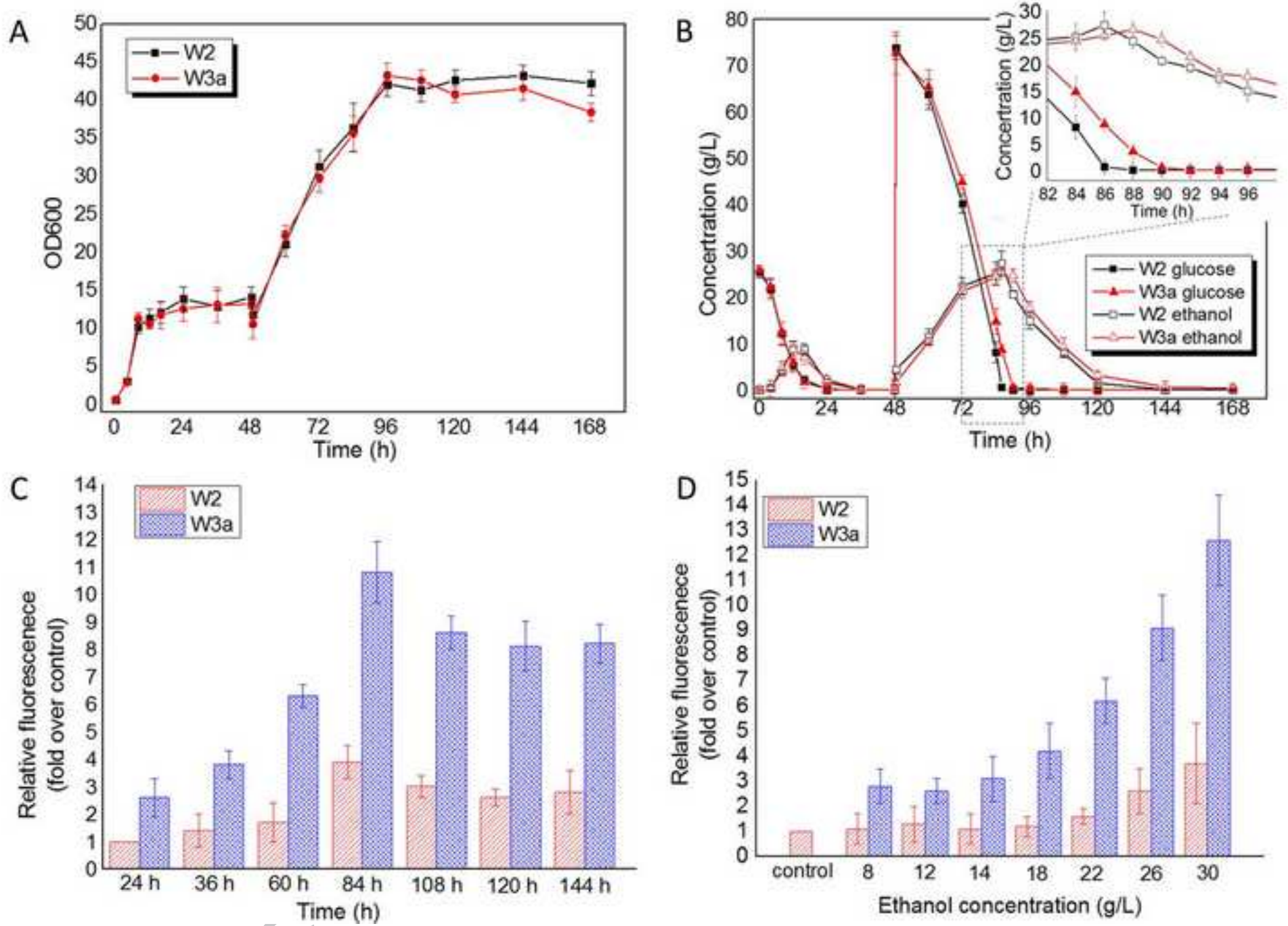


Figure 2

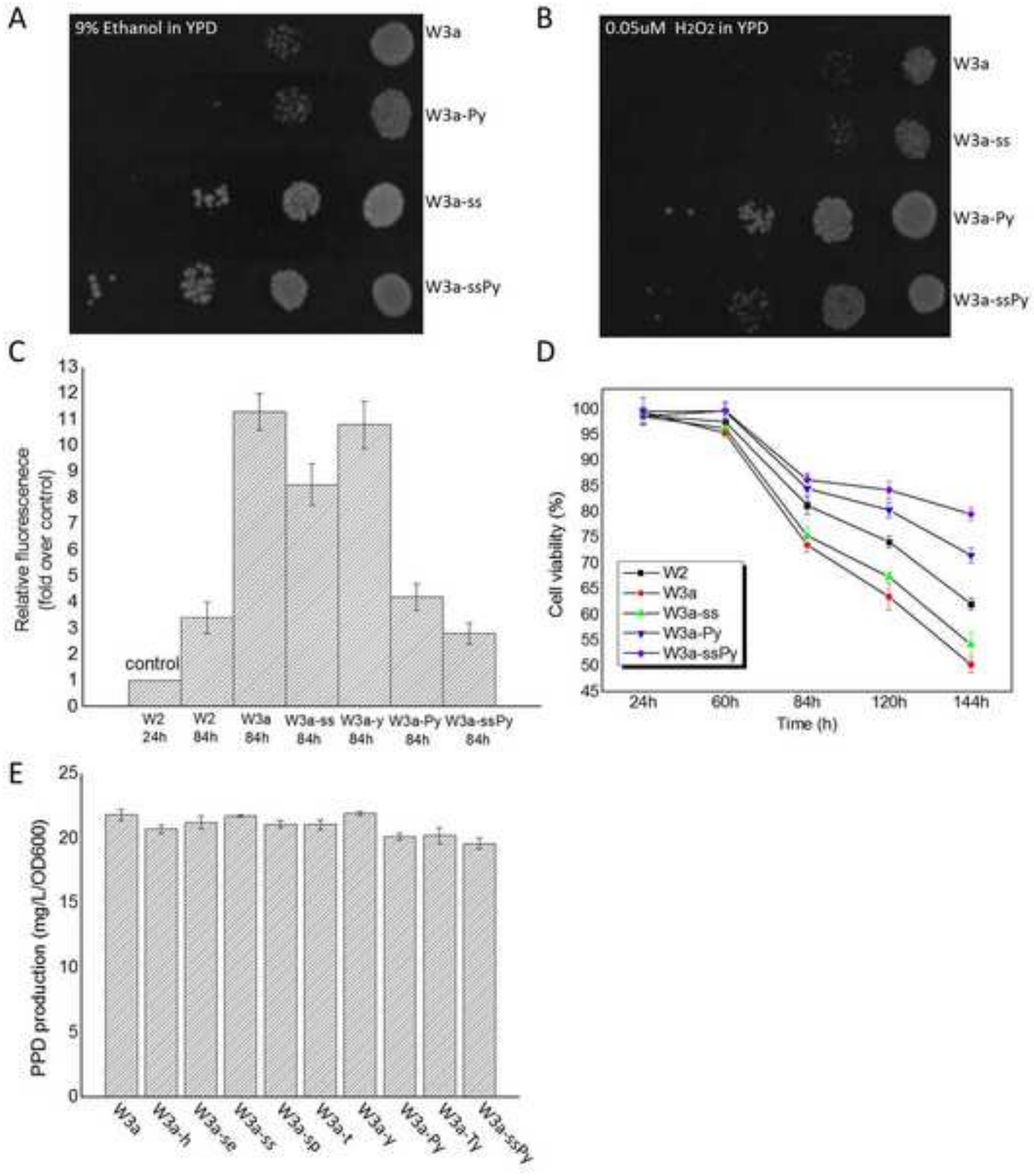


Figure 3

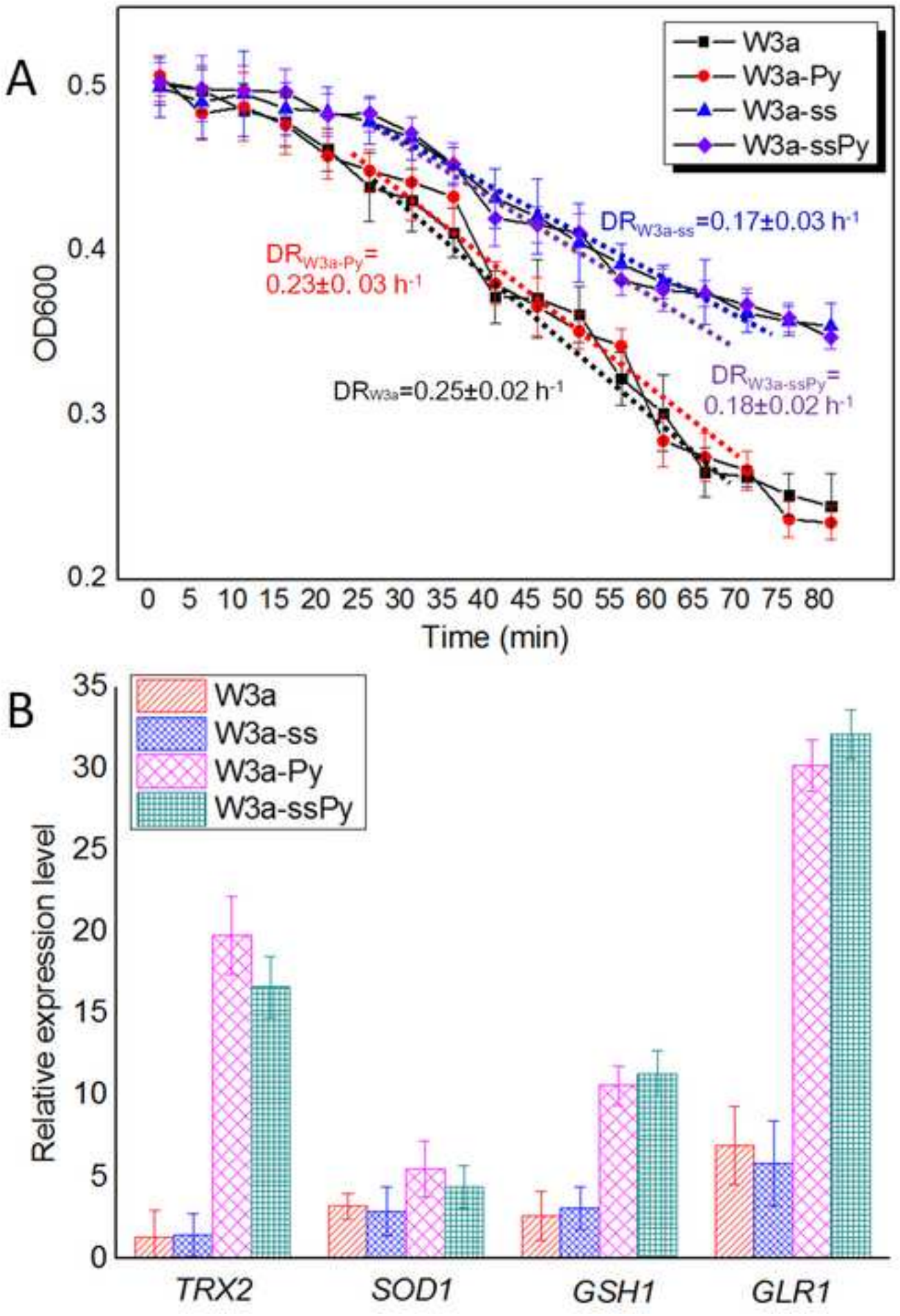


Figure 4

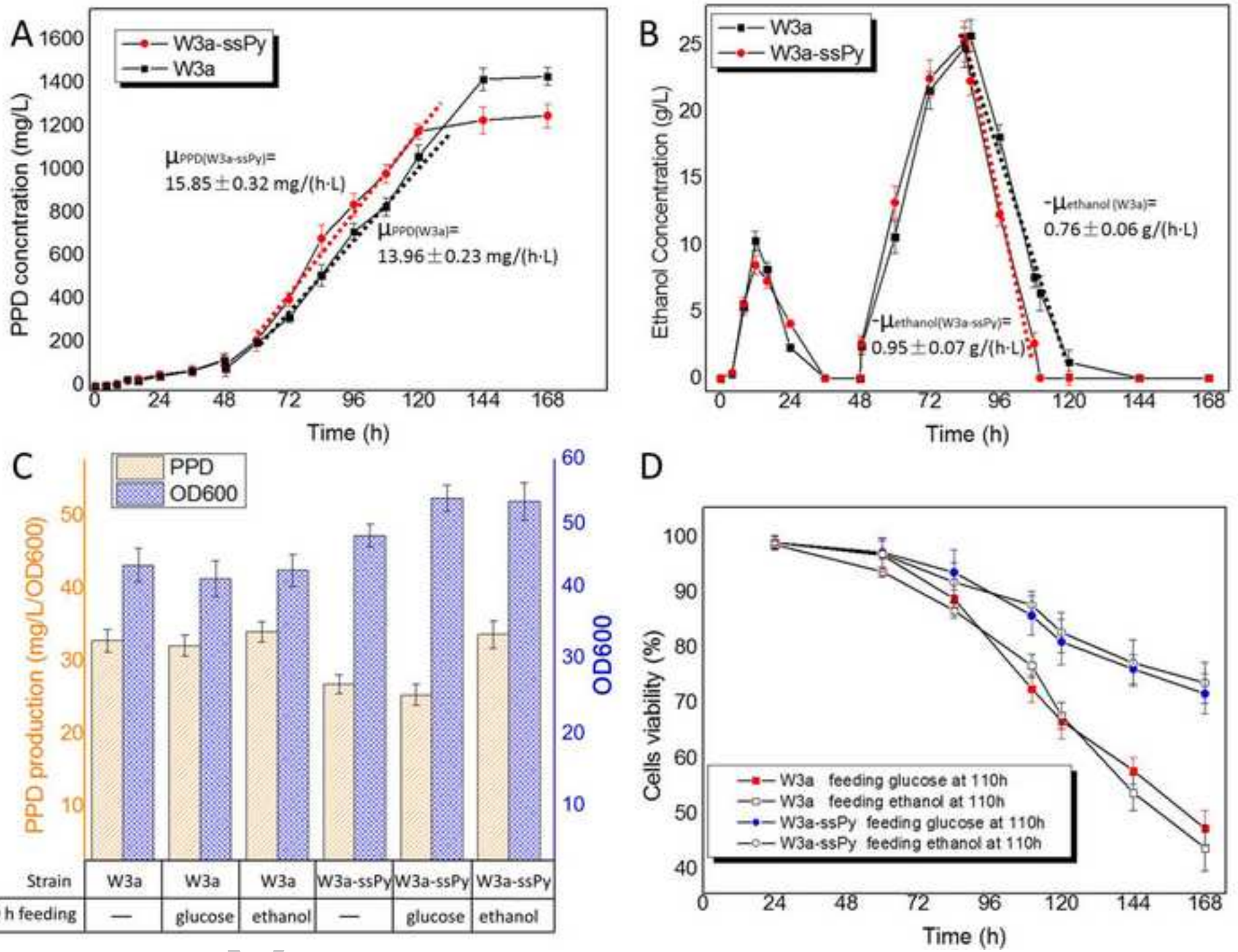
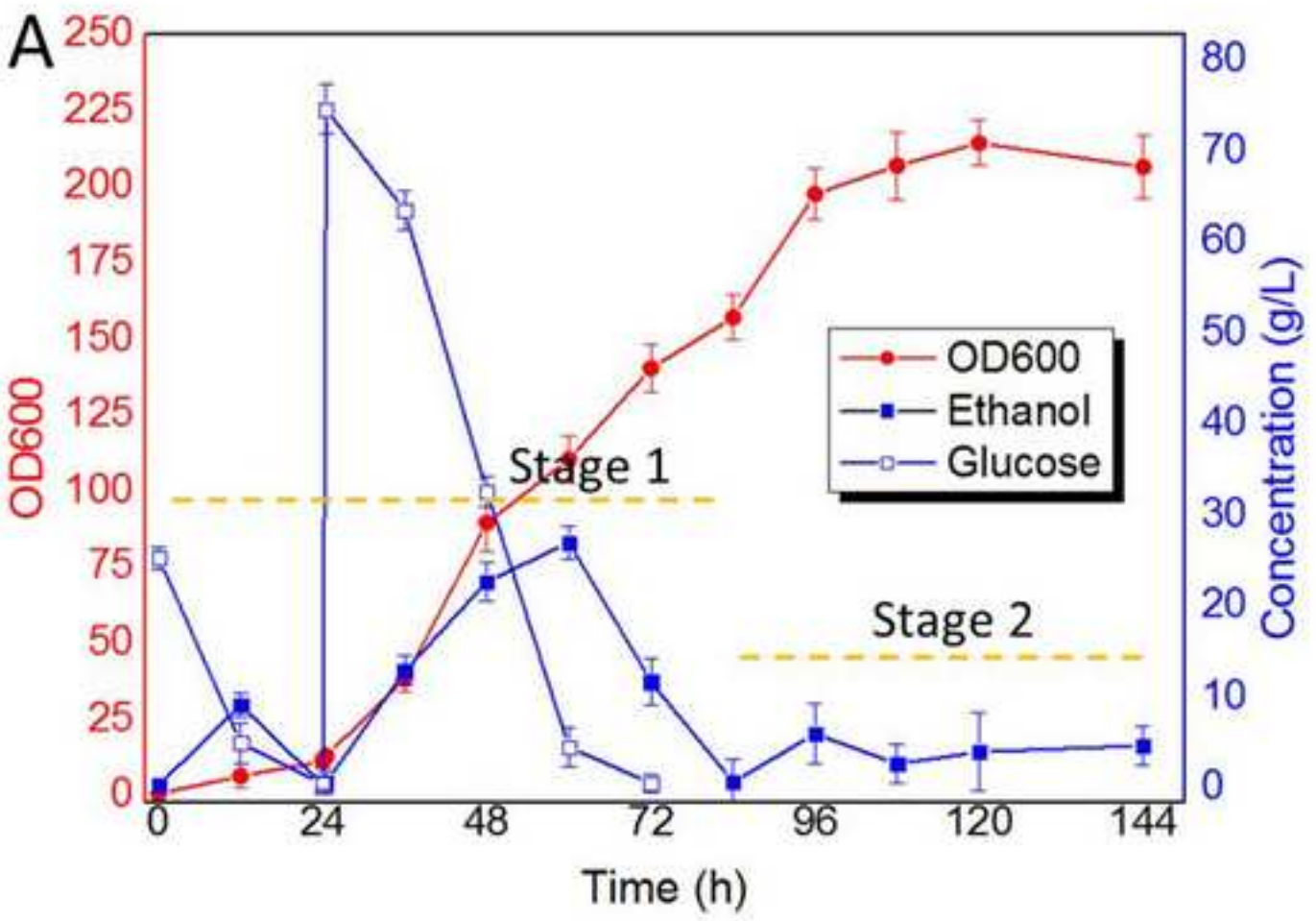


Figure 5



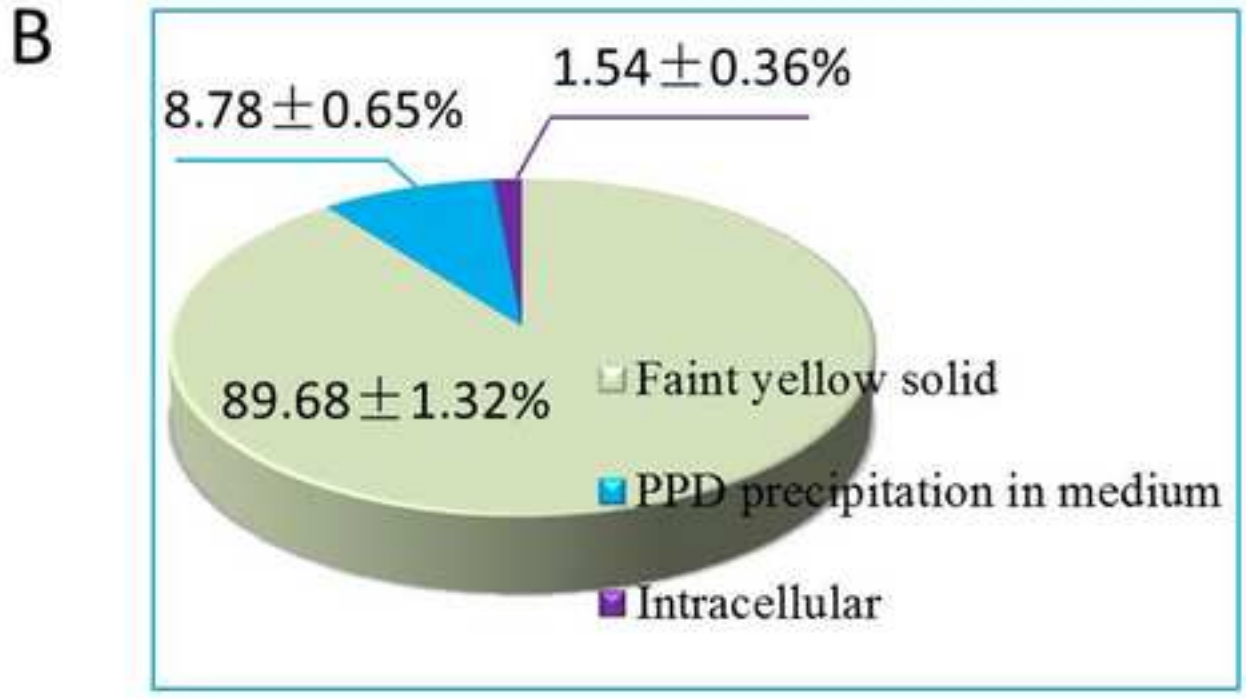


Table 1. Strains used in this study

Strain	Description	Source
W2	Dammarenediol II producting strain	Zhao et al., 2016
W3a	Protopanoxadiol producting strain	Zhao et al., 2016
W3aHU	*URA3* and *His3* markers were deleted using a Cre-expressing plasmid pSH63	this study
W3a-h	*PGK1p-HSP150-ADH1* cassette was inserted into *his3* site of W3aHU	this study
W3a-sp	*PGK1p-SPI1-ADH1* cassette was inserted into *his3* site of W3aHU	this study
W3a-ss	*PGK1p-SSD1-ADH1* cassette was inserted into *his3* site of W3aHU	this study
W3a-se	*PGK1p-SED1-ADH1* cassette was inserted into *his3* site of W3aHU	this study
W3a-t	*PGK1p-TIP1-ADH1* cassette was inserted into *his3* site of W3aHU	this study
W3a-y	*YBP1* cassette was inserted into *ura3* site of W3aHU	this study
W3a-Py	*PGK1p-YBP1* cassette was inserted into *ura3* site of W3aHU	this study
W3a-Ty	*TEF1p-YBP1* cassette was inserted into *ura3* site of W3aHU	this study
W3a-ssPy	*PGK1p-YBP1* cassette was inserted into *ura3* site of W3a-ss	this study

Table 2. Primers used for qPCR.

Primers	**Sequence 5′→3′**
F-ACT1-q	GATTCTGAGGTTGCTGCTTTGG
R-ACT1-q	CGATAGATGGGAAGACAGCACG
F-TRX2-q	CGCTTCTGAATACGACAGTGC
R-TRX2-q	GCAAACTTTTCAATCATTGGTGC
F-SOD1-q	GTTCAAGCAGTCGCAGTGTTAAAG
R-SOD1-q	TCGTAAGAGACAGTGGTTGGC
F-GSH1-q	GGAGACGAGCTTGAGTACATGG
R-GSH1-q	ACTCACATCGTTAGCCTCACAAAG
F-GLR1-q	TCCACGAACACCAAGCATTAC
R-GLR1-q	CTTCAACTAGTAATGTCTTCGCACC

Table 3.
Results of fermentation in 5 L bioreactor. Values represent the sum of intra- and extracellular concentration. * indicate the ratio of PPD to the sum of PPD and DMD.

Batch.NO	OD600	PPD (mg/L/OD600)	PPD (mg/L)	DMD (mg/L)	Ratio*	End time
1	206.6	18.99	3923.6	102.3	97.4%	120 h
2	214.1	19.72	4223.2	134.2	96.9%	144 h
3	212.8	18.87	4105.6	141.6	96.6%	168 h
4	225.3	19.67	4432.3	132.6	97.1%	144 h
5	215.9	19.06	4116.5	163.2	96.2%	144 h

Highlights

•A synergistic effect of P450-CPR uncoupling and ethanol stress on ROS releasing was spotted.

•Cells viability was improved after enhancing ROS and ethanol stress tolerance.

•PPD production of W3a-ssPy reached to 4.25 g/L in 5 L reactor, which is the highest yield reported.

•This work made the production of PPD possible by fermentation instead of phytoextraction.