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Mitochondrial fusion and fission are involved in stress tolerance of *Candida glabrata*

Shubo Li[1,2,3,4], Liming Liu[1,3,4*] and Jian Chen[1,3*]

Abstract

Background: Recently, cell tolerance toward environmental stresses has become the major problem in the development of industrial microbial fermentation. Acetoin is an important chemical that can be synthesized by microbes. Its toxicity was investigated using *Candida glabrata* as the model in this study.

Results: A series of physiological and biochemical experiments demonstrated that the organic solvent acetoin can inhibit cell growth by increasing intracellular reactive oxygen species (ROS) production and inducing damage to mitochondria and cell apoptosis. Integrating RT-PCR experiments, the genes *fzo*1 and *dnm*1 were overexpressed to regulate the balance between mitochondrial fusion and fission. Enhancement of mitochondrial fusion was shown to significantly increase cell tolerance toward acetoin stress by inhibiting ROS production and increasing the intracellular adenosine triphosphate (ATP) supply, which was also demonstrated by the addition of citrate.

Conclusions: Regulating mitochondrial fusion-fission may be an alternative strategy for rationally improving the growth performance of eukaryotes under high environmental stress conditions, and also expands our knowledge of the mechanisms of cell tolerance through the processes of energy-related metabolic pathways.

Keywords: Acetoin; ATP supply; *Candida glabrata*; Cell tolerance; Environmental stress; Mitochondrial fusion-fission

Background

With the development of metabolic engineering, microorganisms have been engineered to produce many important bulk chemicals, such as ethanol, acetone, and butanol. However, most of these are toxic to cells as they can damage cell membranes and walls and interfere with essential physiological processes [1]. Thus, improving cell tolerance has become a major challenge for microbial fermentation. To this end, genomic tools (transcriptomics, proteomics, and metabolomics) have been applied to investigate microbial responses to various solvents, and a number of strategies have been exploited to avoid solvent toxicity. Recently, a visual summary of cellular responses directed toward overcoming solvent stress and enhancing survivability was compiled [2-4], as follows: (i) metabolic detoxification, converting toxic compounds into less harmful chemicals; (ii) expression of heat shock proteins (HSP),

assisting in protein folding and preventing aggregation; (iii) use of proton motive force and associated energy production; (iv) molecular efflux pumps, exporting solvents to the extracellular space; and (v) changes in cell membrane composition and biophysics, combating the fluidizing effects of solvents. But, interestingly, most of these involve energy-dependent processes, indicating that energy and energetic processes play crucial roles in protecting cells against environmental stress. For example, enhancing glucose transport and catabolism favors increased energy production and compensates for the energy expended in relieving stress in prokaryotes and eukaryotes [5-7]. Therefore, engineering the energy-generation machinery could be an alternative approach to improving cell tolerance toward environmental stress.

Mitochondria, as the power plants of the cell, can supply most of its energy through oxidative phosphorylation, and play an important role in maintaining cellular functions, such as the citric acid cycle and cell apoptosis [8,9]. As highly dynamic organelles, mitochondria maintain a balance between frequent cycles of fusion and fission, which tightly regulate mitochondrial number, morphology, and functions under ever-changing physiological conditions [10-12]. The

* Correspondence: mingll@jiangnan.edu.cn; jchen@jiangnan.edu.cn
[1]State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[3]The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China
Full list of author information is available at the end of the article

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mitochondrial fusion process can be divided into three events: docking, fusion of the outer membrane, and fusion of the inner membrane. An increase in fusion events can result in mitochondrial inner connected networks, which are protective against apoptotic sensitivity and promote survival under conditions of stress [13]. In yeast, the mechanisms of mitochondrial fusion and fission have been identified and characterized. The fusion apparatus requires the activity of the large mitochondrial GTPase mitofusin (MFN), consisting of three core components, Fzo1, Ugo1, and Mgm1, of which Fzo1 is the key component regulating lipid bilayer fusion of mitochondrial outer membranes [11]. However, for mitochondrial fission, the core apparatus needs another large GTPase, a dynamin-related protein that includes three components, Dnm1, Fis1, and Mdv1, of which Dnm1 is required for mitochondrial fission and apoptosis [12,14]. Therefore, mitochondria have the ability to coordinate a balance between fusion and fission to handle cellular and environmental stressors [15,16].

Candida glabrata, a haploid multivitamin auxotrophic yeast, is an important strain used for industrial production of pyruvate [17] and α-ketoglutaric acid [18]. Additionally, *C. glabrata* was also used to produce malate [19] and acetoin [20], which can serve as a high value-added platform for the food, pharmaceutical, and chemical industries as a member of the C4-dicarboxylic acid family. However, high concentrations of acetoin were found to inhibit cell growth [20], and increasing the tolerance of cells may be favorable to improving the fermentative performance of a strain. To this aim, *C. glabrata* was used to study the effects of acetoin stress on cellular physiological characteristics (such as reactive oxygen species (ROS) and cell viability), and the balance between mitochondrial fusion and fission was also regulated to improve cellular physiological characteristics, thus enhancing cell robustness. These results demonstrated that enhancing mitochondrial fusion to increase the adenosine triphosphate (ATP) supply may be a novel approach to improving cell tolerance toward environmental stress.

Methods

Strains and plasmids

Escherichia coli JM109 was purchased from Invitrogen (Carlsbad, CA, USA) and used for plasmid construction. *C. glabrata* Δ*ura*3 was derived from *C. glabrata* CCTCCM 202019 (multivitamin auxotroph) and used as the receptor for gene overexpression. More information about the plasmids and strains is given in Table 1.

Plasmid construction and transformation

The primers used in this work are listed in Additional file 1: Table S1, and standard cloning and bacterial transformations were performed according to Sambrook and Russell [21]. The genes *fzo*1 and *dnm*1 were amplified by polymerase chain reaction (PCR) from genomic DNA of *S. cerevisiae* (CEN.PK2-1C), and then inserted into the desired plasmid multi-cloning sites. In all cases, PCR was performed using TaKaRa *Pyrobest* DNA Polymerase (Takara Bio Inc, Shiga, Japan). All genes were sequenced to ensure correct identify of the insert prior to transformations. Yeast strains were transformed using the lithium acetate method [22].

Culture medium and conditions

During construction, strains were grown in complex (YPD) medium consisting of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. All engineered strains were screened on synthetic complete (SC) medium consisting of 20 g/L glucose, 7 g/L urea, 5 g/L KH_2PO_4, 0.8 g/L $MgSO_4 \cdot 7H_2O$, 3 g/L sodium acetate, and 15 g/L agar, at pH 6.0. They were fermented in a medium (medium A) consisting of 100 g/L glucose, 3 g/L urea, 7 g/L KH_2PO_4, 0.8 g/L $MgSO_4 \cdot 7H_2O$, and 5 g/L sodium acetate in shake-flask culture (200 rpm, 30°C) using $CaCO_3$ as the buffering agent. The inoculum size and the vitamin solution (0.04 mg/L thiamine-HCl, 0.16 mg/L biotin, 0.4 mg/L pyridoxine-HCl, and 8 mg/L nicotinic acid) were added to all media to constitute 15% *v/v* and 1% *v/v*, respectively. When necessary, different acetoin concentrations were

Table 1 Plasmids and strains used in this study

Strains/plasmids	Relevant characteristics	Reference
Plasmids		
pYX212	2 μm, Amp^R, *URA3*, P$_{TPI}$	Lab collection
pYX212-*fzo*1	Harboring the gene of *fzo*1 from *Saccharomyces cerevisiae* CEN.PK2-1C	This study
pYX212-*dnm*1	Harboring the gene of *dnm*1 from *S. cerevisiae* CEN.PK2-1C	This study
Strains		
C. glabrata CCTCC M202019	Multivitamin (thiamine, biotin, nicotinic acid and pyridoxine) auxotroph	[21]
C. glabrata Δ*ura*3	The mutant derived from *C. glabrata* CCTCC M202019	[19]
C-pYX	*C. glabrata* Δ*ura*3 (pYX212)	This study
C-Fzo1	*C. glabrata* Δ*ura*3 (pYX212- *fzo*1)	This study
C-Dnm1	*C. glabrata* Δ*ura*3 (pYX212- *dnm*1)	This study

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added to the culture. Transformed *E. coli* JM109 cells were grown at 37°C in Luria-Bertani (LB) medium containing 100 μg/mL ampicillin.

Spotting assay for evaluation of acetoin tolerance

To evaluate microbial tolerance, a spotting assay was applied in the presence of acetoin [23]. First, the cells were incubated overnight in SC medium with shaking (200 rpm, 30°C), and then they were collected and resuspended by centrifugation (10,000×g, 20 s) in sterilized water. Second, the suspensions were serially diluted to an OD_{660} of 1×10^0, 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, and 1×10^{-8}, and then spotted (5 μL each) onto agar plates containing different acetoin concentrations. The plates were sealed with vinyl plastic tape to prevent evaporation of acetoin and incubated at 30°C.

Analytical methods

The optical absorbance at 660 nm (A_{660}) was converted to dry cell weight (DCW) according to the following formula [17]: A_{660} DCW = 1: 0.23 (g/L).

Cell survivability

When the cells were incubated to OD_{660} = 1.0, some of cultures were diluted in sterile deionized water and spread evenly onto SC plates with varied acetoin concentrations (0%, 10%, and 15%), and the colony forming units (cfu) were determined after 24 h of incubation. Cell survival was expressed as survivability (cfu %), and defined as below:

$$\text{Cell survivability} = \frac{\text{The number of colonies on acetoin-containing agar plate}}{\text{The number of colonies on non-acetoin-containing agar plate}} \times 100\%;$$

Physiological parameters

When the cells had been incubated to the exponential phase (OD_{660} = 2.0), different acetoin concentrations were added and continual cultivation proceeded for 24 h (200 rpm, 30°C). Cells were then collected and washed three times with sterilized water. Afterwards, the OD was adjusted to 1.0 before measuring the following physiological parameters (parameters were expressed as mean values with standard error of the mean of at least three independent experiments):

(1) Mitochondrial membrane potential ($\Delta\Psi_m$): $\Delta\Psi_m$ was measured using Rh123 (Sigma, Shanghai, China) as described previously [24]. Cells were collected and washed twice with phosphate buffer saline (PBS), and the pellets were incubated with 500 μM Rh123 in the dark for 10 min at room temperature. Then cells were washed with PBS three times, and the suspensions were measured using flow cytometry (FCM, BD Biosciences, Shanghai, China).

(2) ROS level: cells were collected and prepared using the ROS Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China), and ROS production was detected via flow cytometry.

(3) ATP production: cells were collected and prepared according to the protocol of the ATP Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China), and ATP levels were measured with a luminometer. Protein concentrations were determined using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China).

(4) Cell apoptosis: cell apoptosis was measured by flow cytometric analysis using the FACS Calibur (BD Biosciences, Shanghai, China). Fluorescence emission was measured through a 500/50-nm bandpass filter for Rh123-labeled cells, and through a 660/16-nm bandpass filter for PI-labeled cells. Propidium iodine (PI, Sigma, Shanghai, China) staining was used to monitor cell membrane integrity. Samples (500 μL) were incubated with 3 μL PI stock solution (1 mg/mL) for 5 min at room temperature in the dark, followed by microscopy and flow cytometry, and cell viability was calculated by measuring PI fluorescence on a log scale. Before measuring cell apoptosis and viability using staining with flow cytometry, Rh123/PI dual staining was quantified using samples from fresh cultures with or without acetoin treatment. A minimum of 10,000 events were analyzed per sample at a low flow rate. CELL QUEST software was used for data acquisition and analysis [25].

Transcriptional analysis

Total RNA isolation was carried out using an RNAprep pure Plant Kit, and reverse transcription (cDNA synthesis) was performed according to the protocol of the Prime-Script®RT reagent kit Perfect Real Time (Takara Bio Inc, Shiga, Japan). Quantitative real-time PCR (RT-PCR) was done using the β-ACTIN gene as the internal control, and the primers used in RT-PCR are given in Additional file 1: Table S1. Each sample was tested in triplicate in a 96-well plate (Bio-Rad Corp, Hercules, CA, USA).

Statistical analysis

All experiments were carried out in triplicate, and the results are expressed as mean ± standard deviation. SPSS 18 (SPSS Statistics 18.0, SPSS Institute, Inc., Chicago, IL, 2010, USA) was used for one-way analysis of variance and canonical correlation analysis (CCA), and significant differences ($P < 0.05$) among means were determined by the least significant difference test.

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Results

Tolerance of *C. glabrata* toward acetoin stress

To determine the toxicity of the important chemical acetoin, a spotting assay on agar plates containing different acetoin concentrations was used. As shown in Figure 1a, *C. glabrata* grew on agar plates containing 6 g/L of acetoin, but was significantly inhibited by 15 g/L of acetoin. Furthermore, cell tolerance was also evaluated in shake-flask culture, and it was found that cell growth decreased by 7.5% (6 g/L), 19.8% (12 g/L), 44.4% (15 g/L), and 59.2% (18 g/L), respectively, compared to the control conditions (without addition of acetoin) (Figure 1b). Therefore, 6 g/L of acetoin had a slight effect on cell growth, which was consistent with the spotting assay results.

Effects of acetoin stress on cellular physiological characteristics

To investigate the toxic effects of acetoin on cells in depth, the parameters cell viability and intracellular ROS were measured under conditions of acetoin stress. Cell viability was reduced to 22% after treatment with 18 g/L acetoin (Figure 2a), while intracellular ROS production increased to 32.5 from 14.1 compared to the control conditions (without addition of acetoin) (Figure 2b). Furthermore, double staining with Rh123 and PI, which could discriminate intact, apoptotic, and necrotic cells, was applied to observe cell apoptosis by flow cytometry. As shown in Figure 3, Rh123/PI double fluorescent staining with flow cytometry generated a contour diagram with three distinct regions: R1, necrotic cells; R2, apoptotic cells; R3, intact cells. When the concentration of acetoin increased from 0 to 18 g/L, the percentage of necrotic cells (R1) increased slightly from 0.8% to 2.15%, but the percentage of apoptotic cells (R2) increased significantly to 96.48%. Correspondingly, the percentage of intact cells (R3) decreased markedly from 99.22% to 1.37%. Therefore,

the levels of cell apoptosis were effectively increased during high levels of acetoin stress. These results demonstrate that acetoin can dramatically change the levels of cell viability and intracellular ROS, and induce cell apoptosis and death, and then significantly inhibit cell growth.

Roles of mitochondrial fusion and fission in tolerance toward acetoin stress

In yeast, mitochondria are the major source of ROS production, and the processes of mitochondrial fusion-fission are closely related to cell apoptosis [26]. Therefore, the gene expression levels of *fzo*1 and *dnm*1 were measured by RT-PCR under different acetoin conditions. As shown in Figure 4a, the expression levels of *fzo*1 and *dnm*1 were dramatically enhanced, increased by 3.4- and 1.8-fold, respectively, at 12 g/L of acetoin. To confirm these results, engineered C-Fzo1 and C-Dnm1 overexpressing *fzo*1 and *dnm*1, respectively, were constructed using the strain C-pYX (harboring the plasmid pY212) as the control. As shown in Figure 4b, no distinct differences were observed in cell growth between C-pYX, C-Fzo1, and C-Dnm1 in the absence of acetoin stress. But under high acetoin stress, the engineered strain C-pFzo1 exhibited superior tolerance compared to the strain C-pYX, as it grew well on an agar plate containing 18 g/L of acetoin, and even grew in the presence of 20 g/L acetoin. In contrast, the strain C-Dnm1 was significantly inhibited in the presence of 15 g/L acetoin, showing lower tolerance than strain C-pYX. Therefore, increasing mitochondrial fusion improved cell tolerance, whereas increasing mitochondrial fission had a negative effect on cell tolerance toward acetoin stress.

Effects of mitochondrial fusion-fission on physiological characteristics

To elucidate the mechanisms by which mitochondrial fusion-fission regulate cell tolerance, the effects of
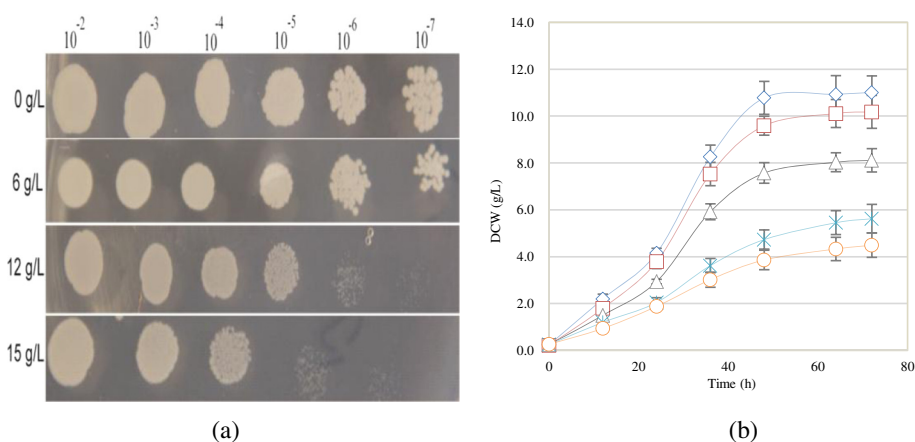

Figure 1 Cell tolerance toward acetoin stress. Application of a spotting assay **(a)** and time course of cell growth (DCW) in shake-flask culture with acetoin added at 0 h **(b)**. Error bars represent mean values and standard deviations of three independent experiments.

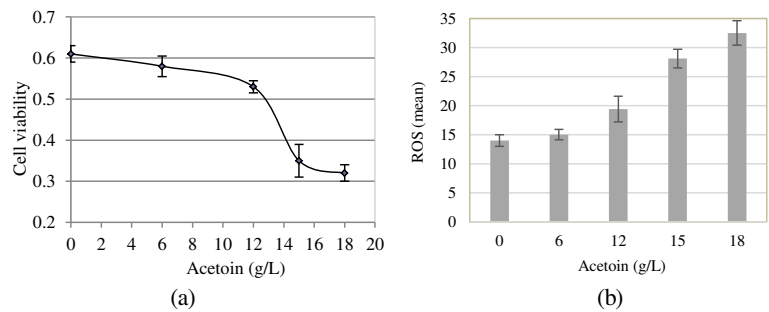Li *et al. Bioresources and Bioprocessing* (2015) 2:12

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Figure 2 Effects of different acetoin concentrations on cell viability (a) and intracellular ROS production (b). Error bars represent mean values and standard deviations of three independent experiments.

mitochondrial fusion-fission on cellular physiological characteristics were further investigated. As shown in Figure 5, the intracellular environment significantly changed due to the expression of mitofusin in the engineered strains. Compared to the control conditions (without addition of acetoin), some different characteristics were observed in engineered strains cultured with 15 g/L of acetoin, as follows. (1) Strain C-Fzo1 effectively inhibited the production of intracellular ROS (increased by only 69.3%), which was far lower than that produced by strains C-pYX (increased by 144%) and C-Dnm1 (increased by 180%) (Figure 5a). (2) Strain C-Fzo1 significantly

maintained cell survivability and ATP production, as these parameters decreased by 44.3% and 40.7%, respectively, to values that were higher than those of strains C-pYX (decreased by 64.4% and 69.7%, respectively) and C-Dnm1 (decreased by 75.7% and 76.1%, respectively) (Figure 5b,c). Furthermore, strain C-Fzo1 more effectively maintained the balance of mitochondrial membrane potential ($\Delta\Psi m$, with a decrease of 18.6%) than strain C-Dnm1 ($\Delta\Psi m$ decreased by 48.2%), which may be due to lower ROS production and higher ATP formation (Figure 5d). These results demonstrate that the enhancement of mitochondrial fusion could improve the ATP supply and decrease ROS production to
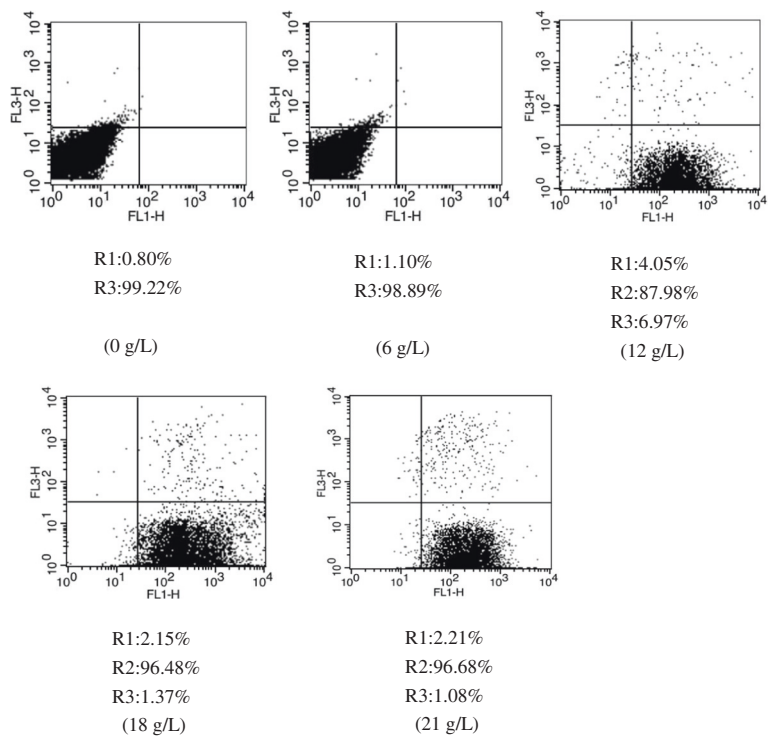

Figure 3 Contour diagram of the flow cytometry of *C. glabrata* cells under the conditions of different acetoin concentrations. FL1-H, Rh123 staining; FL3-H, PI staining. Region 1 (R1), percentage of necrotic cells; region 2 (R2), percentage of apoptotic cells; region 3 (R3), percentage of intact cells.
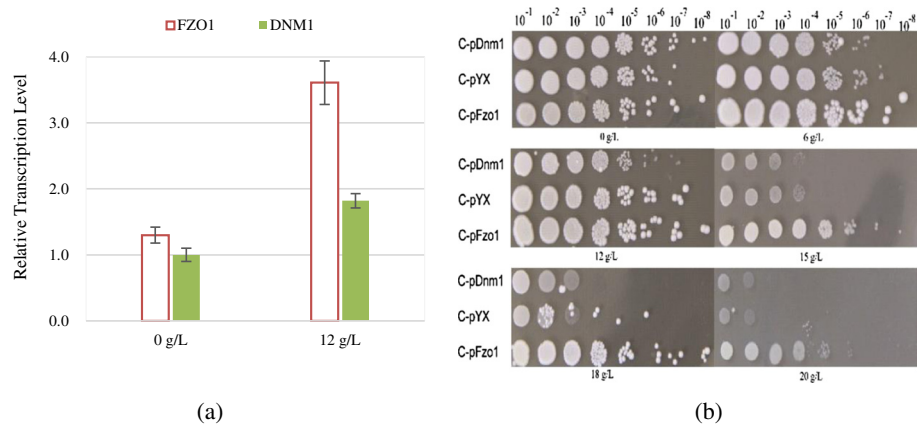
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Figure 4 Roles of mitochondrial fusion-fission in cell tolerance toward acetoin stress. (a) The gene expression levels of *fzo*1 and *dnm*1. **(b)** Spotting assay of the engineered strains.

maintain the balance of $\Delta\Psi m$, and thus increase cell survivability and cell tolerance against acetoin stress.

Effect of citrate on *C. glabrata* adaptation to acetoin stress

According to the results of a previous study [27], 50 nmol/ L of sodium citrate was added to determine its effects on cell growth and ATP production of the wild-type strain under different acetoin conditions. As shown in Figure 6a, compared to the control conditions (without addition of sodium citrate), sodium citrate effectively improved intracellular ATP supply, increasing ATP production by 18.8% (6 g/L of acetoin), 48.9% (12 g/L of acetoin), and 11.7% (15 g/L of acetoin). Correspondingly, cell tolerance of acetoin stress was also enhanced, so that cell growth increased by 18.2% (6 g/L of acetoin), 30.1% (12 g/L of acetoin), and 9.5% (15 g/L of acetoin) (Figure 6b). Therefore, improving the supply of cellular ATP could indeed increase cell tolerance toward acetoin stress.
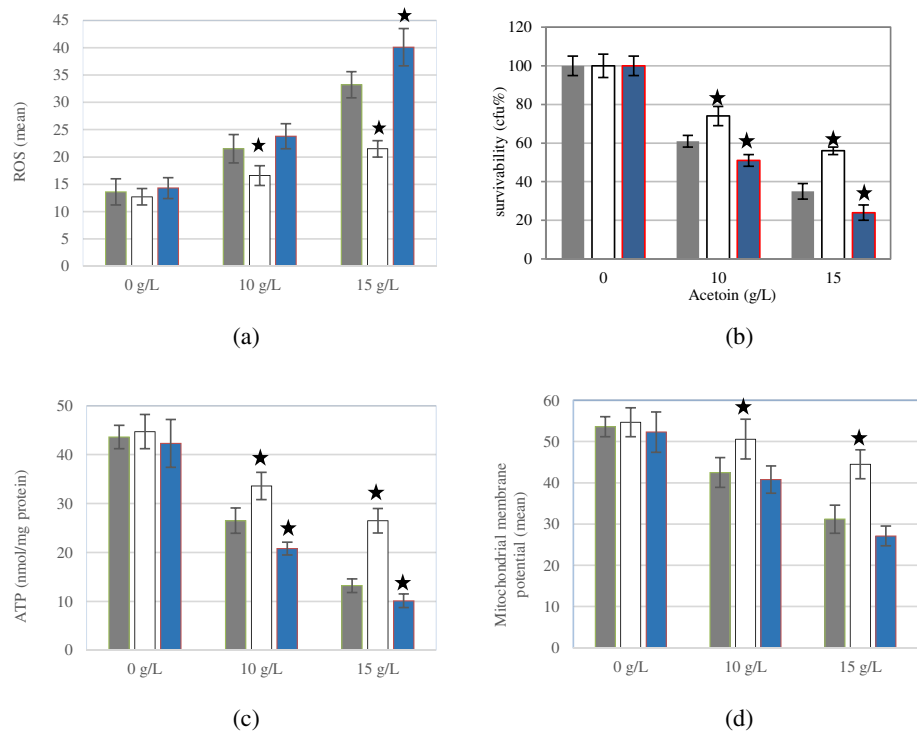

Figure 5 Effects of acetoin stress on cell physiological characteristics in the different engineered strains. (a) ROS generation. **(b)** Cell viability. **(c)** ATP production. **(d)** $\Delta\Psi m$. Error bars represent mean values and standard deviations of three independent experiments (black square, C-pYX; white square, C-Fzo1; blue square, C-Dnm1) (*$P < 0.05$ vs. the group of strain C-pYX).

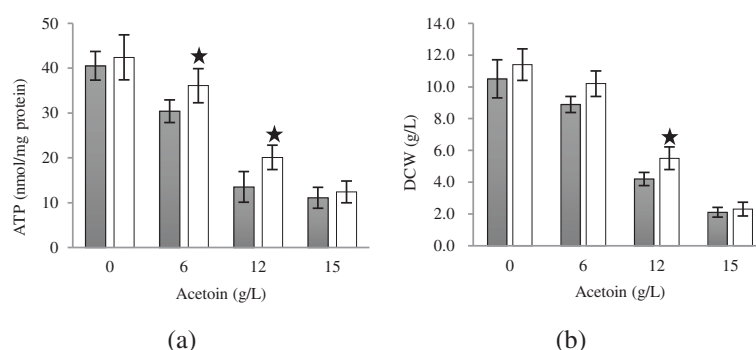Li *et al. Bioresources and Bioprocessing* (2015) 2:12

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Figure 6 Effects of citrate addition on intracellular ATP production (a) and cell growth (b). (black square, without citrate addition; white square, with citrate addition). Error bars represent mean values and standard deviations of three independent experiments (*$P < 0.05$ vs. the group without citrate addition).

Discussion

In cells, mitochondria continually generate ROS as the byproduct of electron transport during oxidative phosphorylation. If ROS production increases it can damage mitochondrial components (such as proteins, lipids, and DNA) and then weaken metabolism and further increase ROS generation, causing a 'vicious downward spiral' of ROS generation and damage accumulation [28,29]. Additionally, intracellular ROS can participate in signaling effects to control the activity of protein kinases and phosphates and regulate gene expression, and thus induce cell apoptosis and cell death [30,31]. Here, the results indicated that acetoin stress could significantly increase intracellular ROS formation, and then decrease cell viability and induce cell apoptosis (Figures 2 and 3). Therefore, we hypothesized that reducing intracellular ROS production may be an alternative route for enhancing cell tolerance toward acetoin stress.

In eukaryotes, mitochondrial fusion-fission is important for maintaining mitochondrial numbers and morphology, and plays a critical role in sustaining functional mitochondria [32,33]. To date, the processes of mitochondrial fusion-fission have been used to investigate and explain cell death induced by different environmental stresses, such as ethanol [34], hyperosmotic [35], and acetic acid stress [36]. In such cases mitochondrial fission could facilitate apoptosis by inducing mitochondrial fragmentation and ROS production, but fusion could compensate the contents of partially damaged mitochondria and maintain energy output to mitigate various stresses. In this study, the processes of mitochondrial fusion-fission were regulated and used to enhance cellular properties to combat acetoin stress. As a result, the engineered strain C-Fzo1 overexpressing mitofusin *fzo*1, which regulates mitochondrial fusion and maintains mitochondrial morphology, could effectively inhibit intracellular ROS formation and increase cell viability, and thus enhance cell tolerance during high levels of acetoin stress.

But, interestingly, enhancement of mitochondrial fusion could also effectively improve ATP production and help maintain the mitochondrial membrane potential ($\Delta\Psi$m), which may be another reason for the high tolerance of strain C-Fzo1. Apoptosis was apparent through measurement of characteristic apoptotic indicators, such as decreased $\Delta\Psi$m, production of ROS, and caspase involvement, in which $\Delta\Psi$m became transiently increased upon apoptotic stimulation in yeast cells, resulting in mitochondrial fragmentation, the release of apoptogenic factors including cytochrome c and apoptosis-inducing-factor (AIF) [37,38], and a permanent decrease in $\Delta\Psi$m during subsequent apoptotic processes [39,40]. Therefore, maintaining the mitochondrial membrane potential may be favorable for enhancing the tolerance of cells toward environmental stress. Additionally, citrate was added and this demonstrated that improving the ATP supply had a positive effect on cell growth in the presence of acetoin stress. Actually, mitochondrial fission-fusion has been shown to alter energy requirements to regulate cell components, so that hyperfused mitochondria led to an increase in mitochondrial oxygen consumption and ATP formation that enhanced cell growth [41,42]. Therefore, the results reported here provide an alternative strategy for rationally improving the growth performance of eukaryotes, especially those that are organic solvent producers, under high organic solvent conditions, and also expand our knowledge of the mechanism of organic solvent tolerance through the processes of energy-related metabolic pathways.

Conclusions

In this study, the processes of mitochondrial fusion-fission were regulated and used to inhibit intracellular ROS production, raise the intracellular ATP supply, and maintain the mitochondrial membrane potential, and therefore increase cell tolerance toward acetoin stress. However, mitochondrial fission-fusion can also regulate many other

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physiological functions, such as Ca^{2+} homeostasis [43], metabolite transportation, and signaling exchange [32], to maintain the metabolic stability of mitochondria. Hence, the roles of mitochondrial fission-fusion in influencing the intracellular environment and physiological characteristics require further comprehensive investigation, exploiting some novel strategies to improve the performance of industrial microorganisms in a future study.

Additional file

Additional file 1: Table S1. Primers used for RT-PCR and PCR amplification in this study.

Author details
[1]State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China. [2]College of Light Industry and Food Engineering, Guangxi University, Nanning 530004, China. [3]The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China. [4]Laboratory of Food Microbial-Manufacturing Engineering, Jiangnan University, Wuxi 214122, China.

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