JMB Papers in Press. First Published online Oct 26, 2018
DOI: 10.4014/jmb.1806.06014

Manuscript Number: JMB18-06014

Title: Ethanol induces autophagy regulated by mitochondrial ROS in Saccharomyces cerevisiae

Article Type: Research article

Keywords: Autophagy, ethanol stress, fermentation, hydrogen peroxide, superoxide anion, reactive oxygen specie
Ethanol induces autophagy regulated by mitochondrial ROS in *Saccharomyces cerevisiae*

Hongjuan Jing, Huanhuan Liu, Zhang Lu, Jie Gao, Haoran Song, Xiaorong Tan

College of Biological Engineering, Henan University of Technology, Zhengzhou 450001, China

Running head: Ethanol induces autophagy in *Saccharomyces cerevisiae*.

Corresponding author:

Hongjuan Jing
Tel: +86-371-67756513; E-mail: hjjing@haut.edu.cn.

Xiaorong Tan
Tel: +86-371-67756513; E-mail: tanxr2012@gmail.com.
Abstract

Ethanol accumulation inhibited the growth of *Saccharomyces cerevisiae* during wine fermentation. Autophagy and the release of reactive oxygen species (ROSs) were also induced under ethanol stress. However, the relation between autophagy and ethanol stress was still unclear. In the study, expression of autophagy genes *ATG1* and *ATG8* and production of ROS under ethanol treatment in yeast were measured. The results showed that ethanol stress very significantly induced expression of *ATG1* and *ATG8* genes and the production of peroxide hydrogen (H$_2$O$_2$) and superoxide anion (O$_2^-$). Moreover, the *atg1* and *atg8* mutants aggregated more H$_2$O$_2$ and O$_2^-$ than the wild type yeast. In addition, inhibitors of the ROS scavenging enzyme induced expression of *ATG1* and *ATG8* genes through increasing the levels of H$_2$O$_2$ and O$_2^-$. Oppositely, glutathione (GSH) and N-acetylcystine (NAC) decreased the *ATG1* and *ATG8* expression by reducing H$_2$O$_2$ and O$_2^-$ production. Besides that, rapamycin and 3-methyladenine caused an obvious change in autophagy levels and simultaneously altered the release of H$_2$O$_2$ and O$_2^-$. At last, inhibitors of mitochondrial electron transport chain (mtETC) increased the production of H$_2$O$_2$ and O$_2^-$ and also promoted expression levels of the *ATG1* and *ATG8* genes. In conclusion, ethanol stress induced autophagy which was regulated by H$_2$O$_2$ and O$_2^-$ derived from mtETC. In turn, the autophagy contributed to the elimination H$_2$O$_2$ and O$_2^-$.  

Key words: autophagy; ethanol stress; fermentation; hydrogen peroxide; superoxide anion; reactive oxygen species
Introduction

In order to remove dysfunctional and unneeded constituents and to recycle intracellular nutrients, macroautophagy (hereafter referred to as autophagy) encapsulated and transferred cytoplasmic material to the vacuole or lysosome for degradation\(^1\). Autophagy-related genes (ATGs) originally were identified in the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*)\(^2\). The most important of ATGs was an ubiquitin-like protein ATG8 that attached to lipid phosphatidylethanolamine (PE) in the outside of emerging phagophore. The ATG8-PE adduct contributed to expand and seal the vesicle and recruit specific cargo\(^3-4\). Therefore, upregulation of *ATG8* expression was implicated the induction of autophagy\(^5\). Moreover, the initiation of autophagy was regulated by Ser/Thr kinase ATG1 and its accessory regulator ATG13 in fungi\(^6-10\). It had been proven that the expression of *ATG1* was also the index of autophagy level\(^11\).

During the early stage of fermentation, sufficient nitrogen helped the proliferation and growth of yeast. Then, the further consumption of nitrogen resulted in the sugar fermentation. Therefore, nitrogen limitation was very common in winemaking. It was well known that nitrogen starvation could induce autophagy\(^12-13\). However, autophagy was induced early in wine fermentation in a nitrogen-replete environment\(^14\). About the function of autophagy during fermentation, it had been reported that recycling of iron via autophagy was critical for the transition from glycolytic to respiratory growth\(^15\). Therefore, the function of autophagy and the exact factors inducing autophagy were still unknown during fermentation.

More and more studies had shown that reactive oxygen species (ROSs) were associated with fermentation\(^16-19\). ROS was possible part of the yeast response to a
variety of stress factors occurred during must fermentations\(^{[20]}\). ROS, mainly including
the superoxide anion (O\(_2^−\)) and hydrogen peroxide (H\(_2\)O\(_2\)), were generated by cells
during normal metabolism\(^{[21]}\). Many researches had shown that ROS had vital role in
regulating autophagy\(^{[21-23]}\). However, which molecule of ROS regulated autophagy
was still in debated. For example, it had been proven that H\(_2\)O\(_2\) had ability of
activating autophagy\(^{[21]}\). However, Chen et al had shown that O\(_2^−\) could induce
autophagy\(^{[24]}\). In addition, other ROS except O\(_2^−\) possibly induced autophagy\(^{[12]}\).
Therefore, the exact ROS molecule regulating autophagy still needed to be researched
in the future.

Ethanol production was likely to remain the most important biotechnological
products in the future, such as alcohol, wine, sake, beer and so on \(^{[25]}\). Therefore,
ethanol accumulation in the culture broth became a significant stress factor during
fermentation. Although \textit{S. cerevisiae} was highly ethanol tolerance, high ethanol still
inhibited cell growth and viability\(^{[26-28]}\). However, mechanism of ethanol tolerance
was still unclear. The aim of current study was to obtain the new insights on the
response of yeast under ethanol stress conditions and clarified complicated
relationship between ROS and autophagy during fermentation. To achieve these aims,
the ROS production and the expression of \textit{ATG8} or \textit{ATG1} genes were evaluated in
wild type and mutants yeast strains growing on medium with ethanol.
Materials and methods

Strain and maintenance medium

The wine yeast strain BY4742 (S. cerevisiae) was supplied by Pro. Zhiwei Huang of East China University. The mutants atg1 and atg8 were purchased from company (Invitrogen Carlsbad, CA). The mutants were constructed by hygromycin stripe homologous replacement the mutation genes of ATG1 or ATG8 and selected by yeast peptone dextrose agar (YPD) medium with 200μg/ml G418. Wild type yeasts could not growth on YPD medium containd G418 whereas mutant cells could survival. The mutants were preserved on YPD medium containing G418 in order to prevent back mutation. The wild type yeast maintained at 4°C on slants of YPD medium contained (g liter⁻¹): glucose 20, peptone 10, yeast extract 5, and agar 20. Fresh cells grew on YPD slants for 24 h were used in all experiments.

Fermentation conditions

For all experiments, starter cultures were prepared by growing the yeast cells overnight in 250-ml flasks containing 100 ml of YPD medium. The flasks were incubated at 30°C in an orbital shaker set at 180 rpm. The experimental cultures were inoculated with 5×10⁵ CFU ml⁻¹ of starter culture. Fermentation was carried out in 500-ml flasks filled to two-thirds of their volume and maintained at 30°C in an orbital shaker at 180 rpm, according to the methodology[12]. Distilled water as control, 10% ethanol by adding absolute ethyl alcohol was as ethanol stress. 100 mM 2-methoxyestradiol (2-ME) was stored dimethylsulfoxide (DMSO) and final concentration was 100 µM. 1 M 3-amino-1,2,4-triazole (3-AT) was stored in double distilled water and final concentration was 2 mM. 10 mM rapamycin (Rapa) dissolved in ethanol and stored in TritonX-100 and final concentration was 5 µM. 3-methyladenine (3-MA) was stored in PBS solution and final concentration was 10
µM. 2.5 mM glutathione (GSH) and 10 mM N-acetyl-cysteine (NAC) were both dissolved in double distilled water. 100 mM antimycin A (Anti A) and rotenone (Rote) were both dissolved in DMSO and final concentration were both 5 mM. Each flask was closed with a rubber stopper. Prior to sampling, the flasks were stirred to ensure homogeneity.

**Assessment of cell death**

Cell death was assessed by propidium iodide (PI) (Sigma, US) vital staining as described elsewhere with minor adaptations. Briefly, yeast (10⁶ cells ml⁻¹) was stained by 20 µM PI for 10 min at 37°C and then observed by fluorescence microscope. The fluorescence intensity were measured by fluorescence 96-well microplate (excitation wavelength 540 nm, emission wavelength 590 nm). The death of cells was with high red fluorescence.

**Assessment of intracellular H₂O₂ and O₂⁻**

H₂O₂ was monitored with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma, US) essentially as described elsewhere. DCFH-DA didn’t fluoresce but could transmembrane freely. The dye could be hydrolyzed by esterase to 2, 7-dichlorofluorescin (DCFH) which was arrested in an actively respiring cell. DCFH was oxidized by ROS to a fluorescent compound DCF in cytoplasm. To detect O₂⁻, dihydroethidium (DHE) (Sigma, US) was used as a probe. Briefly, yeast (10⁶ cells ml⁻¹) was stained by 4 µM DHE for 10 min at 30°C in dark. The fluorescences of DCF and DHE were detected by fluorescence microscope and Fluorescence-activated cell sorter (FACS) analysis.

FACS analysis was carried out with an FACS Calibur flow cytometer (Becton, Dickinson and Company, US) equipped with an argon ion laser emitting a 488-nm beam at 15 mW. The green and red fluorescence were collected through a 488-nm
blocking filter. Green fluorescence and red fluorescence were used to detect by a 550-nm/long-pass dichroic mirror with a 525-nm/band-pass filter and a 590-nm/long-pass with a 620-nm/band-pass filter, respectively. An acquisition protocol was defined to measure green fluorescence (FL1 log) and red fluorescence (FL2 log) on a 4-decade logarithmic scale. Data (20,000 cells per sample) were analyzed with the cell quest pro included in the System II acquisition software for the Flow Jo software.

**qRT-PCR**

Wild type yeast was treated different reagent with ethanol. RNA was obtained from the sample which was periodically collected. RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) standard procedures. Heat shock treatment (15 min at 42°C followed by 3 min at 95°C) was for cellular disruption. Total RNA (250 ng) was reverse transcribed using a SuperScript III Platinum Two-Step real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) kit with SYBR green from Invitrogen. One microliter of the reverse-transcribed RNA was used as a template to amplify the genes, using primers to the ATG8 gene (sense, 5'-TTGCTGACAGGTTCAAGAATAGG-3; antisense, 5'-ATCAACGCCGCAGTAGGTG-3), ATG1 gene (sense, 5'-TACTGTGCTCTTGGGGACCTA-3; antisense, 5'-CGGACGCTAACTGCTGTAATA-3) and the ACT1 gene (sense, 5'-GGATTCTGAGGTTGCTGCTTT-3; antisense, 5'-TGACCCATACCGACCATGATA C-3). The expression of the ATG8 and ATG1 genes were assessed by qRT-PCR in a Stepone plus (ABI). Results were normalized to the reference gene ACT1. The data were analyzed by applying the Livak method or the $2^{-\Delta\Delta C_T}$ method$^{[31]}$. The method was as follows: $\triangle\triangle C_T = (C_T(\text{target gene}) - C_T(\text{reference gene})) \text{ test} - (C_T(\text{target gene}) - C_T(\text{reference gene})) \text{ calibrator}$, where $C_T$ is threshold cycle.
Statistical analysis

Data were reported as mean values of at least three independent assays and presented as means ± standard deviations (SD). Statistical analyses were carried out using a Student’s t test. P values of less than 0.05 or 0.01 were considered statistically significant, very significant which were shown as “*” and “**” at the tops of the columns in the figures.
Results

Ethanol stress induced autophagy

Ethanol was the main metabolites of *S. cerevisiae* during the fermentation process[25]. In order to confirm the effect of ethanol stress on autophagy, the expression levels of *ATG1* and *ATG8* genes were measured by qRT-PCR. Based on the preliminary experiment, the peak expression value of *ATG8* and *ATG1* was at 4h. Therefore, expression of *ATG8* and *ATG1* genes was detected at 4h. The results showed that the expression levels of *ATG1* and *ATG8* genes were very significantly increased during ethanol stress (P < 0.01) (Fig.1). In detail, the expression levels of *ATG1* and *ATG8* were respectively increased to 153.5 % and 252.6 % by ethanol stress, respectively (Fig.1). Therefore, we confirmed that ethanol stress indeed induced autophagy.

Autophagy contributed to survival of *S. cerevisiae* from ethanol stress

The above results revealed that ethanol stress markedly induced autophagy. In order to clarify the role of autophagy during ethanol stress, the death rate of the wild type, *atg1* and *atg8* strains were assessed by PI staining. The results showed that there was no difference between the wild type and the mutant strains during their growth on YPD medium (Fig.2). However, ethanol stress increased cell death rate of all strains (Fig.2). Compared with wild type yeast, the cell death of *atg1* and *atg8* mutants was dramatically enhanced by ethanol stress. Therefore, this indicated that autophagy protected yeast cells from ethanol stress.

Ethanol induced production of H$_2$O$_2$ and O$_2^-$ in *S. cerevisiae*

To evaluate whether ROS accumulation was accompanied by ethanol stress, ROS production were measured by fluorescence staining and flow cytometry. Production of H$_2$O$_2$ and O$_2^-$ was reached the highest at 2 h according to the preliminary experiment.
Therefore, production of \( H_2O_2 \) and \( O_2^- \) was detected at 2 h. The production of \( H_2O_2 \) and \( O_2^- \) was both induced by ethanol stress for 2 h (Fig 3A). \( H_2O_2 \) production in \( atg1 \), \( atg8 \) mutants was higher than that in wild type yeast under ethanol treatment for 24 h. In addition, the \( atg1 \) mutant had the highest \( H_2O_2 \) concentration (Fig 3B). Similarly, the \( O_2^- \) contents in mutant cells were also higher than that in wild type yeast cells (Fig 3C). However, the difference in \( O_2^- \) production between wild type and mutants was not greatly different than that observed in \( H_2O_2 \). Therefore, These results demonstrated that ethanol stress contributed to the accumulation of ROS and autophagy had ability of eliminating ROS.

**Autophagy induced by ethanol stress depended on ROS**

The above results had shown that ethanol not only induced the expression of \( ATG1 \) and \( ATG8 \) but also enhanced the production of \( H_2O_2 \) and \( O_2^- \). In addition, accumulating evidence had shown that moderate ROS as signal molecules regulated autophagy\(^{[12,21,23]}\). Therefore, in order to clarify the relation of autophagy and ROS, reductants were used to change ROS levels. Obviously, both GSH and NAC reductants decreased very significantly the production of \( H_2O_2 \) and \( O_2^- \) under ethanol stress (Fig. 4A and 4B). The production of \( H_2O_2 \) was reduced to 55.4% and 44.0% by GSH and NAC (Fig. 4B). GSH and NAC decreased the production levels of \( O_2^- \) to 43.5% and 29.2% (Fig. 4B). Simultaneously, GSH and NAC markedly decreased the gene expression of \( ATG1 \) and \( ATG8 \) (Fig. 4C). For example, expression of \( ATG1 \) and \( ATG8 \) was decreased to 54.7% and 38.8% by GSH. NAC lowered \( ATG1 \) and \( ATG8 \) expression to 55.6% and 19.3%. Besides in wild type, GSH and NAC also decreased the production of \( H_2O_2 \) and \( O_2^- \) in \( atg1 \) and \( atg8 \) mutants(Fig S1).

In spite of moderate ROS acting as a signal, excess ROS could oxidize nearby biological macromolecules including DNA, proteins and lipids\(^{[32-36]}\). In order to
protect themselves from oxidative damage, cell used enzymes to eliminate ROS in cell, such as catalase (CAT) in cytoplasm and manganese superoxide dimutase (Mn-SOD) in mitochondria\textsuperscript{37-39}. Therefore, 2-ME and 3-AT were used as the inhibitors of Mn-SOD and CAT, to increase of O$_2^-$ and H$_2$O$_2$ levels in cells. The results had shown that 2-ME increased the production of H$_2$O$_2$ and O$_2^-$ (Fig. 5A and Fig 5B). 3-AT only induced the levels of O$_2^-$ to 159.0% but had no effect on the contents of H$_2$O$_2$ (Fig 5B). 2-ME and 3-AT both activated the expression of \textit{ATG1} and \textit{ATG8} genes under ethanol stress (Fig. 5C). In detail, the expression of \textit{ATG1} and \textit{ATG8} was elevated to 216 % and 183.9 % by 2-ME. 3-AT increased the expression levels of \textit{ATG1} and \textit{ATG8} to 185.6% and 152.1 %, respectively. The production of H$_2$O$_2$ and O$_2^-$ in \textit{atg1} and \textit{atg8} mutants also induced by 2-ME and 3-AT (Fig S2).

To further verify that ROS regulated autophagy in yeast under ethanol stress, the inhibitor of autophagy (3-MA) and the inducer of autophagy (Rapa) were added in the medium under ethanol stress. the results showed that Rapa very significantly promoted the production of H$_2$O$_2$ and O$_2^-$ in wild type yeast (Fig.6A and Fig 6B) and evoked the gene expression of \textit{ATG1} and \textit{ATG8} (Fig. 6C). On the contrary, 3-MA dramatically decreased only the content of H$_2$O$_2$ (Fig.6A and Fig 6B) and reduced the gene expression of \textit{ATG1} and \textit{ATG8} (Fig. 6C). Therefore, autophagy was dependent on content of H$_2$O$_2$ under ethanol stress. In mutants, Rapa and 3-MA both promoted the production of H$_2$O$_2$ and O$_2^-$ under ethanol stress (Fig S3). Rapa and 3-MA did not change the levels of autophagy in the mutants. Accumulation more of H$_2$O$_2$ and O$_2^-$ by Rapa and 3-MA maybe attributed to their alternative function.

**ROS induced by ethanol stress mainly origined from mitochondria**

Since we found that ROS was implicated in autophagy under ethanol stress, the source of ROS was also explored in this study. The respiratory complexes I, II, and III...
of the mitochondrial electron transport chain (mtETC) had been reported to be the
major production sites of O$_2^-$\cite{40-41}. Anti A and Rote, as the inhibitors of complex III
and complex I of the mtETC, were used to increase ROS in mitochondria. The results
had shown that Anti A and Rote both undoubtly increased production of H$_2$O$_2$ and
O$_2^-$ (Fig. 7A and Fig. 7B) and promoted the expression of ATG1 and ATG8 genes
under ethanol stress (Fig. 7C). In detail, production of H$_2$O$_2$ was increased to 212.8%
and 257.4% by Rote and Anti A, respectively. Rote and Anti A added production of
O$_2^-$ to 225.0% and 299.2%, respectively. Expression of ATG1 and ATG8 was
increased to 496.4 % and 431.9 % by Anti A. Rote added ATG1 and ATG8 expression
to 348.5 % and 299.3 %. These findings indicated that ROS derived from the mtETC
regulated autophagy under ethanol stress. Therefore, ROS accumulation in yeast
attribulated to H$_2$O$_2$ and O$_2^-$ derived from the mtETC. In the mutants, Anti A and Rote
also increased production of H$_2$O$_2$ and O$_2^-$ (Fig S4). The results further proved that
production of H$_2$O$_2$ and O$_2^-$ mainly was from the mtETC under ethanol stress.
**Discussion**

The budding yeast had to escape from nitrogen deficient conditions during most alcoholic fermentations. And it was well known that autophagy could be induced by deficient nitrogen during fermentation\cite{12-13,42}. However, Piggott et al. had demonstrated that autophagy was induced at the early stage of wine fermentation in a nitrogen-replete environment\cite{14}. Therefore, it was suggested that autophagy might be triggered by other factors that arose during early stage of the fermentation. Besides that, it had been proven that autophagy was induced in second-fermentation yeasts during sparkling wine production\cite{43}.

Cost-effective ethanol production depended on rapid and high yielding conversion of carbohydrate to ethanol. Therefore, ethanol accumulation in the culture broth appeared at the early stage of fermentation. It was well known that the yeast had property of ethanol tolerance. However, the mechanism of the ethanol tolerance was not clear. In the current study, the results showed that autophagy was induced by ethanol stress (Fig. 1). And compared with *atg1* and *atg 8*, wild type contributed to cell survival from ethanol stress (Fig. 2). Therefore, yeast was likely survived from ethanol injury by means of increasing the levels of autophagy to clear away the damaged organelles.

**Ethanol stress induced ROS in *S. cerevisiae***

*CTT1*, encoding a kind of cytosolic CAT, was found to be highly expressed only in ethanol-tolerant sake yeast mutants or after exposure to ethanol\cite{44}. Therefore, eliminating of ROS by CTT1 helped sake yeast survival from ethanol stress. Otherwise, ROS was normally induced during fermentation\cite{18-19}. However, exact relationship of ROS and ethanol stress was still unclear. In this study, ROS production in wild type yeast (Fig. 4, 5, 6, 7) and mutant cells (Fig S1, S2, S4) was induced by
ethanol stress. However, the role of ROS was still unknown under ethanol stress.

Compared to wild type yeast, the \textit{atg1} and \textit{atg8} mutants had higher production of H$_2$O$_2$ and O$_2^-$ under ethanol stress (Fig. 3). These results were in agreement with previous reports. For instance, it had been shown that the mutants \textit{atg2} and \textit{atg5} accumulated high levels of H$_2$O$_2$\cite{45}. Thus, autophagy was in favor of eliminating ROS under ethanol stress. In addition, \textit{atg1} and \textit{atg8} mutants had higher cell death rate than wild type yeast (Fig.2). These findings suggest that wild type yeast markedly decreased cell death under ethanol through eliminating ROS by autophagy.

\textbf{ROS derived from mtETC regulated autophagy under ethanol stress.}

Mounting evidence suggested that ROS might play a role in the control of autophagy\cite{21-23,46-47}. And we had proven that ethanol activated production of H$_2$O$_2$ and O$_2^-$ (Fig.3A) and autophagy (Fig.1). In addition, GSH and NAC decreased autophagy through decreasing release of H$_2$O$_2$ and O$_2^-$ under ethanol stress (Fig.4). In agreement, NAC had been reported to reduce ethanol-induced autophagy\cite{48} and also to decrease salinomycin-induced autophagy\cite{49}. On the contrary, in our study 2-ME induced autophagy by means of increasing production of H$_2$O$_2$ and O$_2^-$ whereas 3-AT increased autophagy probably just through production of O$_2^-$ (Fig.5). Therefore, our results revealed that H$_2$O$_2$ and O$_2^-$ regulated autophagy levels in the yeasts under ethanol stress.

Moreover, Rapa promoted the production of H$_2$O$_2$ and O$_2^-$ and increased the expression of \textit{ATG1} and \textit{ATG8} genes (Fig.6). However, 3-MA decreased the content of H$_2$O$_2$ and lowered the expression of \textit{ATG1} and \textit{ATG8} genes (Fig.6). The difference was that both Rapa and 3-MA enhanced production of H$_2$O$_2$ and O$_2^-$ in \textit{atg1} and \textit{atg8} mutants under ethanol stress (Fig S3). Rapa and 3-MA did not affect the level of autophagy in mutants. Alternative functions of Rapa or 3-MA maybe associated with
the increased accumulation of $H_2O_2$ and $O_2^-$. Although ROS has been previously reported to be implicated in autophagy, which kind of ROS that played a crucial regulatory role was still disputed. Chen et al. considered that $O_2^-$ was the main kind of ROS inducing autophagy$^{[24]}$. However, Mendes-Ferreira et al reported that all kinds of ROS except $O_2^-$ had vital roles in regulating autophagy$^{[12]}$. There were also some reports showed that autophagy was induced by $H_2O_2^{[21,46]}$. In our study, $H_2O_2$ and $O_2^-$ both participated in regulating autophagy in the yeast under ethanol stress.

Mitochondrion was the main source of ROS$^{[40-41]}. In the current study, Anti A and Rote also increased the production of $H_2O_2$ and $O_2^-$ in atg1 and atg8 mutants under ethanol (Fig S4). The results proved that $H_2O_2$ and $O_2^-$ were mainly derived from the mtETC under ethanol. Anti A and Rote enhanced the production of $H_2O_2$ and $O_2^-$ and activated the expression of ATG1 and ATG8 genes in wild type (Fig. 7). Therefore, ROS derived from mitochondria had vital roles in regulating autophagy under ethanol stress. Many researches had shown that ROS from mitochondria regulated autophagy or mitophagy$^{[39,50-52]}$. For example, MITA expression modulated autophagy flux through enhancing mitochondrial ROS by increasing complex-I activity$^{[51]}$. In addition, mitochondrial complex I inhibition triggered a mitophagy-dependent ROS increase$^{[52]}$.

Overall, ethanol stress induced autophagy and increased the production of $H_2O_2$ and $O_2^-$ which were originated from the mtETC although other sources of ROS are not excluded. Under ethanol stress, the high level of $H_2O_2$ and $O_2^-$ remarkably induced autophagy. Subsequently, the autophagy contributed to the elimination of $H_2O_2$ and $O_2^-$. Ultimately, the autophagy assisted yeast to survive from ethanol stress during fermentation.
Acknowledgments

This work was supported by projects the National Natural Science Foundation of China (31201409; 31471296), Natural Science Project of Henan Science and Technology Department (162300410175), National Engineering Laboratory for Wheat & Corn Further Processing, Henan University of Technology (NL2016011), and Fundamental Research Funds for the Henan Provincial Colleges and Universities in Henan University of Technology (2016QNJH20).
References


functionality modulating beta-barrel outer membrane protein genes. *Biochimica et Biophysica Acta*. **1857**: 789-798


**Figure legend**

**Fig. 1** Ethanol induced expression of the *ATG1* and *ATG8*. Normalized fold expression levels of the *ATG1* and *ATG8* were evaluated by qRT-PCR in *S. cerevisiae* treated with 10% ethanol for 4 h. *ACT1*, encoded actin, was used as internal reference. Values indicated mean ± standard deviation (n=3). Statistical significance (**, P < 0.01) was determined by a Student’s t test and was shown as the statistic significance between ethanol with control. Con: control, Eth: treated with ethonal.

**Fig. 2** Cell death of mutants was higher than that of wild type under ethanol stress. Wild type, *atg1* and *atg8* treated with 10% ethanol for 2h were stained with PI. Then all strains were analyzed by fluorescence microscope (A) and fluorescence microplate reader (B). Values indicated mean ± standard deviation from (n=6). Statistical very significance (**, P < 0.01) was determined by a Student’s t test and was shown as the statistic significance between ethanol with control. Con: control, Eth: treated with ethonal.

**Fig. 3** Ethanol induced more H$_2$O$_2$ and O$_2^-$ in mutants than that in wild type.

Production of H$_2$O$_2$ and O$_2^-$ in wild type treated with 10% ethanol for 2 h were stained by DCFH or DHE (A). Production of H$_2$O$_2$ (B) and O$_2^-$ (C) in wild type, *atg1* and *atg8* treated with 10% ethanol for 24 h were stained by DCFH (B) or DHE (A). And all strains were analyzed by flow cytometry. Black bar represented 10 um.

**Fig. 4** GSH and NAC decreased production of H$_2$O$_2$ and O$_2^-$ and expression of *ATG1* and *ATG8* genes in *S. cerevisiae* under ethanol stress. A. Production of H$_2$O$_2$ and O$_2^-$ in wild type treated with 10% ethanol for 2h was stained by DCFH or DHE, respectively. Black bar represented 10 um. Relative fluorescence of DCFH and DHE was statisticted in B. Values indicated mean ± standard deviation (n=6). GSH or NAC decreased expression of *ATG1* and *ATG8* (C). Normalized fold
expression levels of the ATG1 and ATG8 were evaluated by qRT-PCR in S. cerevisiae under ethanol stress for 4 h. ACT1, encoded actin, was used as internal reference. Values indicated mean ± standard deviation (n=3). Statistical significance (**, P<0.01) was determined by a Student’s t test.

**Fig. 5** 2-ME and 3-AT increased production of H$_2$O$_2$ and O$_2^-$ and expression of ATG1 and ATG8 genes in *S. cerevisiae* under ethanol stress. A. Production of H$_2$O$_2$ and O$_2^-$ in wild type treated with 10% ethanol for 2h was stained by DCFH or DHE, respectively. Black bar represented 10 um. Relative fluorescence of DCFH and DHE was statisticted in B. Values indicated mean ± standard deviation (n=6). 2-ME and 3-AT increased expression of ATG1 and ATG8 (C). Normalized fold expression levels of the ATG1 and ATG8 were evaluated by qRT-PCR in *S. cerevisiae* under ethanol stress for 4h. ACT1, encoded actin, was used as internal reference. Values indicated mean ± standard deviation (n=3). Statistical significance (**, P<0.01) was determined by a Student’s t test.

**Fig. 6** Rapa and 3-MA regulated production of H$_2$O$_2$ and O$_2^-$ and expression of ATG1 and ATG8 genes in *S. cerevisiae*. A. Production of H$_2$O$_2$ and O$_2^-$ in wild type yeast with 10% ethanol for 2h was stained by DCFH or DHE, respectively. Black bar represented 10 um. Relative fluorescence of DCFH and DHE was statisticted in B. Values indicated mean ± standard deviation (n=6). Rapa and 3-MA regulated expression of ATG1 and ATG8 (C). Normalized fold expression levels of the ATG1 and ATG8 were evaluated by qRT-PCR in *S. cerevisiae* under ethanol stress for 4h. ACT1, encoded actin, was used as internal reference. Values indicated mean ± standard deviation (n=3). Statistical significance (**, P<0.01) was determined by a Student’s t test.

**Fig. 7** Rote and Anti A increased production of H$_2$O$_2$ and O$_2^-$ and expression of
**ATG1 and ATG8 genes in S. cerevisiae.** A. Production of H$_2$O$_2$ and O$_2^-$ in wild type yeast with 10% ethanol for 2h was stained by DCFH or DHE, respectively. Black bar represented 10 um. Relative fluorescence of DCFH and DHE was statisticced in B. Values indicated mean ± standard deviation (n=6). Rote and Anti A increased expression of ATG1 and ATG8 (C). Normalized fold expression levels of the ATG1 and ATG8 were evaluated by qRT-PCR in S. cerevisiae under ethanol stress for 4h. ACT1, encoded actin, was used as internal reference. Values indicated mean ± standard deviation (n=3). Statistical significance (**, P < 0.01) was determined by a Student’s t test.
**Figures**

Figure 1

![Graph showing normalized gene expression of ATG1 and ATG8](image)

- **Con**
- **Eth**

ATG1 and ATG8 gene expression levels are compared between Control (Con) and Ethanol (Eth) conditions. The graph indicates a significant increase in gene expression for ATG8 under Ethanol treatment compared to Control.

*Note: The asterisks (**) indicate statistical significance.*
Figure 2

A

4742  atg1  atg8

Con

Eth

B

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>Eth</th>
</tr>
</thead>
<tbody>
<tr>
<td>4742</td>
<td></td>
<td></td>
</tr>
<tr>
<td>atg1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>atg8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative fluorescence (%)
Figure 3

A

Con

Eth

B

C

sample names: 4742, atg6, atg1

FL1 LOG: DCFH fluorescence/cell

FL2 LOG: DHE fluorescence/cell
Figure 4

A

B

C

ATG1

ATG8

Normalized gene expression

0 1 2 3 4 5 6 7

Con Eth Eth+GSH Eth+NAC

Relative fluorescence(%)
Figure 5

A  

B  

C  

Normalized gene expression

Con Eth Eth+2-ME Eth+3-AT

Absolute fluorescence

Con Eth Eth+2-ME Eth+3-AT

Con Eth Eth+2-ME Eth+3-AT

ATG1  ATG8
Figure 6

A) BF and DCFH images showing cell fluorescence under different conditions: Con, Eth, Eth + Rapa, Eth + 3-MA.

B) Bar graph showing relative fluorescence of DCFH and DHE under Con, Eth, Eth + Rapa, Eth + 3-MA conditions.

C) Normalized gene expression levels of ATG7 and ATG9 under Con, Eth, Eth + Rapa, Eth + 3-MA conditions.
Figure 7

A

B

C

Relative fluorescence (%)

Normalized gene expression

Con Eth Eth + Rote Eth + Anti A

Con Eth Eth + Rote Eth + Anti A

ATG7

ATG8

0 5 10 15 20 25

0 5 10 15 20 25