Changes of trehalose content and expression of relative genes during the bioethanol fermentation by *Saccharomyces cerevisiae*

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Changes of trehalose content and expression of relative genes during the bioethanol fermentation by *Saccharomyces cerevisiae*

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Abstract: Traditionally, trehalose is considered as a protectant to improve the ethanol tolerance of *S. cerevisiae*. In this study, to clarify the changes and roles of trehalose during the bioethanol fermentation, trehalose content and expression of related genes at lag, exponential and stationary phases (i.e., 2, 8, and 16 h of batch fermentation process) were respectively determined. Although yeast cells at exponential and stationary phase had higher trehalose content than cells at lag phase (*P*<0.01), there is no significant difference in trehalose content between exponential and stationary phases (*P*>0.05). Moreover, expression of trehalose degradation-related genes *NTH1* and *NTH2* decreased at exponential phase in comparison to that at lag phase; compared with cells at lag phase, cells at stationary phase had higher expression of *TPS1*, *ATH1*, *NTH1*, and *NTH2* but lower expression of *TPS2*. During the lag-exponential phase transition, down-regulation of *NTH1* and *NTH2* promoted accumulation of trehalose; and to some extent, trehalose might confer ethanol tolerance to *S. cerevisiae* before stationary phase. During the exponential-stationary phase transition, up-regulation of *TPS1* contributed to accumulation of trehalose, and Tps1 protein might be indispensable in yeast cell to withstand ethanol stress at the stationary phase. Moreover, trehalose would be degraded to supply carbon source at stationary phase.

Key words: *Saccharomyces cerevisiae*, bioethanol fermentation process, trehalose, ethanol tolerance, gene expression
Introduction

The serious consequences (energy crisis, global warming and regional disasters) of wide utilization of traditional fossil promoted the demand for alternative renewable energy sources (Goldemberg 2007). As a good alternative to the traditional fossil fuels, renewable bioethanol has little pollution and can ease off the energy crisis (Bai et al. 2008). *Saccharomyces cerevisiae* is an excellent and widely used microbe for industrial production of bioethanol (Bai et al. 2008; Widiastuti et al. 2011).

During the batch bioethanol fermentation, *S. cerevisiae* cells would undergo three phases (i.e., lag, exponential, and stationary phases) (Ding et al. 2009a) and the ethanol would undoubtedly accumulate gradually as fermentation time lasting. Although ethanol is the end product of bioethanol fermentation, the accumulated ethanol itself can also function as a major inhibitor to the growth of *S. cerevisiae* cells and complete bioethanol fermentation (Ding et al. 2009b; Izawa 2010). In the other hand, during the batch fermentation process, cells at various phases would encounter different fermentative environments including the changed ethanol content. Under various fermentative environments, cells at different phases would be characterized by different behaviors, such as changes in metabolisms of carbohydrates (Ding et al. 2009a) or fatty acids (Ding et al. 2009b; Ma and Liu 2010), and so on. To some extent, these changes of cell behaviors including ethanol tolerance could be considered as responses to the changed fermentative environments (Ding et al. 2009a, 2009b; Ma and Liu 2010). Interestingly, our previous result indicated that along with the challenge of accumulated ethanol, the ethanol tolerance of *S. cerevisiae* increased as fermentation time lasting (Dong et al. 2015).

How did the *S. cerevisiae* cells acquire higher ethanol tolerance during the batch fermentation
process? Our previous result demonstrated that during bioethanol fermentation process, yeast cells might remodel membrane and more changeable cell membrane by increasing oleic acid and phospholipids contributed to acquiring higher ethanol tolerance of *S. cerevisiae* cells (Dong et al. 2015). Plasma membranes of the evolved higher ethanol-tolerant strains had higher ratios of C$_{18}$ to C$_{16}$ fatty acids and higher oleic acid (C$_{18:1}$) content than the parental strain (Chen et al. 2014; Tao et al. 2012). Moreover, are there still any other influencing factors on yeast ethanol tolerance or not? At first glance, trehalose, a non-reducing glucose disaccharide and widely present in bacteria, fungi, plants, and many invertebrate cells (Jules et al. 2008), might be worth to be considered as ethanol stress protectant in *S. cerevisiae* cells due to the correlation between trehalose level and cell survival rate under adverse environmental stresses (Petitjean et al. 2015). Numerous previous studies indicated that the trehalose content can reflect the cells tolerance to ethanol stress (Alizadeh and Klionsky 1996; Kwon et al. 2003) and change in response to the environmental variations (Eleutherio et al. 1993). Previous results also implied that the accumulation of trehalose will confer higher ethanol tolerance to *S. cerevisiae* cells (Alizadeh and Klionsky 1996; Kwon et al. 2003). Thehalose in vivo has two main sources. Extracellular trehalose could be transported into cell by Agt1p (Jules et al. 2004) or hydrolyzed to glucose by Ath1p (Nwaka et al. 1996). In addition, intracellular trehalose could also be biosynthesized from glucose by two steps. After trehalose-6-phosphate was catalyzed from UDP-glucose by Tps1p with glucose-6-phosphate, a critical step, trehalose-6-phosphate was dephosphorylated to the trehalose by Tps2p (Elbein et al. 2003). Lastly, intracellular trehalose could be decomposed to glucose under the catalysis of Nth1p, Nth2p (Estruch 2000) or vacuolar Ath1p (Nwaka et al. 1998). However, a recent study suggested that the stress-protecting role of trehalose in *S. cerevisiae* was largely overestimated, and they
indicated that yeast tolerance to various stresses including ethanol stress did not rely on trehalose, but on the trehalose-6P synthase (Tps1) protein (Petitjean et al. 2015). Nevertheless, accumulation of trehalose secondary to higher expression of TPS1 gene also enhanced the ethanol tolerance of Saccharomyces sp. W0, thereby promoting ethanol production (Cao et al. 2014). During the continuous and fed-batch fermentation, trehalose involved genes were similarly upregulated, which included TPS1, TPS2, TSL1, NTH1 and NTH2, except that TPS1 and TPS2 in the third fermentor of continuous fermentation were down-regulated (Li et al. 2010). As ethanol would accumulate with fermentation time lasting and enhance the ethanol inhibition effect on the yeast cell growth, the exact content change of trehalose and roles of trehalose or related genes or proteins in the batch fermentation process are still poorly understood. There were many studies about role of trehalose in ethanol tolerance. For example, in sake brewing, TPS1 and TPS2 expression levels and trehalose content in ethanol tolerance mutant SR4-3 at exponential stage were much higher than those in parent strains. Moreover, after transferred into 10% ethanol YPAD medium, SR4-3 increased the expression of these two genes once more (Ogawa et al. 2000). TPS1 expression of the ale strains was also up-regulated when they were upshifted to 7.5% (v/v) ethanol (Bleoanca et al. 2013). While, rarely researches which combined the yeast fermentation stages with trehalose-related genes were published. A clear insight of trehalose metabolism during the bioethanol fermentation process will contribute to a better understanding of such process and facilitate construction of industrial ethanologenic strains with higher ethanol tolerance (Gibson et al. 2007).

In the present study, to clarify the change and roles of trehalose and related genes during the batch bioethanol fermentation process, S. cerevisiae S288c cells at lag phase, exponential phase
and stationary phase (i.e., 2 h, 8 h, and 16 h of the batch fermentation process, respectively) were chosen to determine the trehalose content. Furthermore, expressions of genes involved in trehalose metabolism at above three phases were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR), thereby contributing to a better understanding of the molecular mechanism underlying the roles of trehalose and related genes during the batch bioethanol fermentation by *S. cerevisiae*.

**Materials and methods**

**Strains, media and culture conditions**

*S. cerevisiae* S288c strain (ATCC number 204508) used in this study was precultured in YPD broth (2% glucose, 1% yeast extract, 2% peptone) for 18 h and then inoculated into high-sugar-YPD broth (20% glucose, 1% yeast extract, 2% peptone) both at 30°C and shaken at 150 rpm in 250 ml cotton-plugged flasks. Besides, the inoculum dose was adjusted to keep the value of OD$_{600}$ of initial inoculated fermentation culture at 0.1.

**Determination of growth curve, pH value, glucose concentration and ethanol content**

The growth curve of *S. cerevisiae* cells during the batch bioethanol fermentation was determined every two hours by measuring the optical density of the culture at 600 nm with a 752 visible spectrophotometer (Shanghai Optical Instrument Factory, Shanghai, China). The experiment was performed in triplicate. The growth rate was calculated using OD$_{600}$ values according to the following equation:
\[ \mu = \frac{(\ln X_2 - \ln X_1)}{(t_2 - t_1)} \]

\( X_1 \) and \( X_2 \) refer to the OD\(_{600}\) values of cell cultures at culture time \((t)\) \( t_1 \) and \( t_2 \) time.

The pH value during the bioethanol fermentation was detected every two hours by FE20-FiveEasy™ pH (METTLER-TOLEDO, Switzerland).

Amount of ethanol of incubation during the bioethanol fermentation was measured using gas chromatography (GC) analysis.

Glucose concentration during the bioethanol fermentation was measured using liquid chromatography (LC) analysis. The determination of pH value and contents of ethanol and glucose were all performed in triplicate.

**Determination of trehalose content**

Twenty milliliters of the \( S. \) cerevisiae S288c cultures were respectively harvested after 2 h, 8 h, and 16 h of incubation and disrupted by ultrasonication in SCIENTZ™ D Noise Isolating Tamber (Ningbo Scientz Biotechnology Co. Ltd., Ningbo, China). Samples were sonicated for 20 min in pulse mode (40 s on and 20 s off) at 20 kHz. After standing for 6 h at room temperature, samples were centrifuged at 5000 rpm for 10 min. The supernatant was filtered through 0.22 \( \mu \)m microporous membrane and used for measurement of trehalose content by liquid chromatography (LC) analysis. LC analysis was performed on a Waters Sugar-Pak1 column (Waters, USA) and a refractive index detector (0.5 ml/min, 80°C). The experiment was performed in triplicate.

**Reverse transcription of total RNA and qRT-PCR analysis**

Expressions of genes involved in the trehalose metabolism were determined in the study. For measurement expression of these genes, samples were harvested from \( S. \) cerevisiae S288c cells...
cultured in the YPD broth fermented for 2 hours, 8 hours, and 16 hours, respectively. Pellets were quickly frozen in liquid nitrogen for RNA extraction or store at -80°C until use. Total RNA used for synthesis of cDNA was extracted according to the manual instruction of Trizol (Invitrogen, USA), and used for synthesis of cDNA. RNA integrity and purity were evaluated by gel electrophoresis and OD$_{260}$/OD$_{280}$ ratio. cDNA was synthesized using the extracted RNA, M-MLV reverse transcriptase and random primer according to the manual instruction (Promega, USA). cDNA from this step was stored at -20°C for using as the template for qRT-PCR. Relative quantification of genes was performed on an Eppendorf Mastercycler® RealPlex\textsuperscript{2}. Primers of selected trehalose metabolism related genes and β-actin (ACT1) were designed using Primer Premier 5 (www.PremierBiosoft.com) with manual editing, and listed in Table 1. For each amplification reaction, a total of 20.1 µl was used consisting of 2 µl cDNA template, 10 µl real SYBR mixture, 0.3 µl of forward and reverse primer (10 µM each), and 7.5 µl H$_2$O. Amplifications were performed after an initial denaturation at 95°C for 10 min followed by 40 cycles of PCR at 95°C for 15 s, at 60°C for 1 min. The melting curve of each PCR product was determined to ensure the specific amplification of the target gene. The mRNA levels of the selected genes were normalized to that of act1 gene in the same sample. The mean fold change in abundance of transcripts was determined according to the method of Livak and Schmittgen (2001). The experiment was performed in triplicate.

Moreover, pearson correlation analysis was performed using SPSS13.0 software to determine the connection between trehalose content and trehalose synthetic genes or degraded genes.

Table 1

Statistical analysis
Data were analyzed by independent-samples T test with SPSS13.0 for Windows, and standard error of the mean (SEM) was used as error bar. Differences showing P-values less than 0.05 were considered statistically significant.

Results

Fermentation time-course data at different phases

During the bioethanol batch fermentation process, as the nutrients were gradually depleted, the growth rate of *S. cerevisiae* cells would decrease continuously after the early exponential phase (Dong et al. 2015). Moreover, ethanol content would undoubtedly accumulate as fermentation time lasting (Supplementary Fig. S1B). With biomass increasing, the cell metabolism lowered the pH of fermentation broth (Supplementary Fig. S1C).

Differences of trehalose content of cells at different phases

Compared with that in the cells at lag phase, the trehalose content of cells at exponential and stationary phases increased obviously (*P*<0.01) (Fig. 1). However, no obvious difference of trehalose content of cells was detected between exponential phase and stationary phase (*P*>0.05) (Fig. 1).

Expression changes of genes related to trehalose metabolism

To further gain the insight of the functions of trehalose played in the fermentation process and ethanol tolerance, changes of transcriptional levels of genes which are respectively responsible for encoding rate-limiting enzymes of synthesis (i.e., *TPS1*, *TPS2*, *TPS3*, and *TSL1*)
and degradation (i.e., \(ATH1\), \(NTH1\), and \(NTH2\)) of trehalose at different fermentation processes were determined by qRT-PCR. Compared with the cells at lag phase, \(S.~cerevisiae\) cells at stationary phase had higher expressions of \(TPSI\) \((P<0.05)\) (Fig. 2A) but lower expression of \(TPS2\) \((P<0.05)\) (Fig. 2B). Compared with the cells at exponential phase, \(S.~cerevisiae\) cells at stationary phase had lower expression of \(TSL1\) \((P<0.05)\) (Fig. 2D). Moreover, the expression of \(TPS3\) almost did not change during the batch fermentation process \((P>0.05)\) (Fig. 2C).

The detected three genes involved in the degradation of trehalose showed almost similar expression pattern during the batch fermentation process. Compared with the cells at lag and exponential phases, \(S.~cerevisiae\) cells at stationary phase had higher expressions of degradation-related genes \(ATH1\), \(NTH1\), and \(NTH2\) \((P<0.05)\) (Fig. 3). Moreover, the expression of \(NTH1\) and \(NTH2\) was down-regulated at exponential phase in comparison to that at lag phase \((P<0.001)\) (Fig. 3B and C).

**Discussion**

Since bioethanol is an ideal alternative to the traditional fossil fuel, researchers have been committed to improve the ethanol yield, but one of the important reasons of unsatisfactory productivity was that ethanol itself is also a strong inhibitor to the \(S.~cerevisiae\) growth and complete bioethanol fermentation (Yang et al. 2012). Yeast cells at different phases are confronted with various contents of ethanol and would be exposed to more ethanol stress as fermentation time...
lasting. However, our previous result indicated that the ethanol tolerance of *S. cerevisiae* increased
as fermentation time lasting (Dong et al. 2015).

Ethanol stress can negatively affect *S. cerevisiae* plasma membrane associated processes (Piper 1995). Cell membrane would be the first assaulting target of ethanol (Ma et al. 2013), and increasing ethanol can also result in variation of the structure and fluidity of the cell plasma membrane (Li et al. 2014), even induce degradation of proteins (Ding et al. 2009b). Furthermore, the normal aging of cells may also cause aggravation of oxidization and degradation of proteins (Nakaya et al. 2014). Therefore, to survive under the increased ethanol stress, a series of cell physiological changes might occur in *S. cerevisiae* cells to stabilize the structure of membrane and proteins (Tibayrenc et al. 2010). Trehalose, which is widely considered as a stress protectant (Cao et al. 2014; Mahmud et al. 2009; Trevisol et al. 2011), has been proposed to facilitate preserving the integrity of plasma membrane and stabilize proteins and other macromolecules as a chemical chaperone during the injury (Mahmud et al. 2010). Based on this viewpoint, trehalose content should increase continuously during the whole batch bioethanol fermentation to function protecting roles in counteracting ethanol stress. In this study, yeast cells at exponential and stationary phases did have higher trehalose content than cells at lag phase (*P*<0.01) (Fig. 1).

However, although ethanol content at stationary phase (i.e., 16 h) increased obviously in comparison to that at exponential phase (i.e., 8 h) (Supplementary Fig. S1B), no difference of trehalose content was observed between exponential phase and stationary phase (*P*>0.05) as expected (Fig. 1). Moreover, our previous work indicated that ethanol tolerance of cell at stationary phase was much higher than that at exponential phase (Dong et al. 2015). Such inconsistency between ethanol tolerance and trehalose content during the exponential
phase-stationary phase transition might preliminarily indicate that trehalose might be not the most indispensable in yeast cell to increase yeast resistance to ethanol stress during that period (Bandara et al. 2009).

To obtain a clear insight of variation and roles of trehalose during bioethanol batch fermentation process, genes involved in trehalose metabolism were chosen to measure the levels of expressions at different phases (Figs. 2, 3). As the bioethanol fermentation progressed from lag phase to exponential phase, trehalose content increased significantly ($P<0.01$) (Fig. 1). Interestingly, in opposition to what was expected, the increase of trehalose content might be not contributed by the up-regulation of TPS1 and TPS2 which are responsible for encoding trehalose biosynthesis enzymes (Apweiler et al. 2013; Lucero et al. 2000). In fact, the expression of four members of trehalose synthetic complex including TPS1 and TPS2 did not change in exponential phase in comparison to that in lag phase ($P>0.05$) (Fig. 2). On the contrary, the down-regulation of NTH1 and NTH2 ($P<0.001$) (Fig. 3B and C) might contribute to the accumulation of trehalose at exponential phase. However, the expression of ATH1 did not change significantly during this transition ($P>0.05$). Nth1p and Nth2p are both neutral trehalases and possess maximal activity at neutral pH value (Kopp et al. 1993), while Ath1p is an acidic trehalase (Alizadeh and Klionsky 1996). Moreover, the pH value during the bioethanol batch fermentation is reduced from about 6.0 to about 4.0 (Supplementary Fig. S1C). Taken together, the decreased pH value combined with increased ethanol stress might induce the down-regulation of neutral trehalases encoding genes NTH1 and NTH2. As for the acidic trehalase encoding ATH1, though a slight expression decline was detected at this transition, the acidic fermentative environment at this phase might facilitate ATH1 to avoid a significant decrease of expression at exponential phase. As no correlation
between trehalose and the expression of any trehalose synthetic gene (i.e., TPS1, TPS2, TPS3, and TSL1) (Fig. 4) or ATH1 (P>0.05) (Fig. 5A), the negative correlation between trehalose content and expression of NTH1 or NTH2 during lag phase-exponential phase transition (P=0.006, r=-0.935 for NTH1; P=0.001, r=-0.969 for NTH2) (Fig. 5B and C) also confirmed the promoting effect of down-regulation of NTH1 and NTH2 on the accumulation of trehalose.

As the bioethanol fermentation progressed from exponential phase to stationary phase, trehalose content did not change significantly but was still higher than that at lag phase (P<0.01) (Fig. 1). During this transition, the up-regulated TPS1 accounted for the continuously accumulated trehalose. The trehalose-6-phosphate (Tre6P) synthase TPS1, which catalyzes the conversion of glucose to Tre6P, is shown to be crucial for the biosynthesis of trehalose (Bell et al. 1998). However, the trehalose-6-phosphate phosphatase TPS2, which catalyzes the final step of trehalose biosynthesis (from Tre6P to trehalose), might do not play key function during this transition (Gounalaki and Thireos 1994; Petitjean et al. 2015). Of course, the exact mechanism of up-regulation of TPS1 underlying the accumulation of trehalose at stationary is still needed further evaluation in the future, because there was no significant difference of trehalose content between the last two stages. Therefore, trehalose might not play the most important role in ethanol tolerance of S. cerevisiae at stationary phase. Moreover, Tps1 protein might have more important functions in preventing energy depletion through regulation of energy homeostasis as an example of the ‘moonlighting proteins’, which is often vital in withstanding adverse fermentative environments, independent from its enzymatic role in biosynthesis of trehalose (Petitjean et al.)
Considering our previous work which showed that ethanol tolerance of cells at exponential phase was much higher than that at lag phase (Dong et al. 2015), we thought that it was Tps1p that conferred cells more tolerance at transition of exponential-to-stationary phase. In addition, expression level of TPS1 did not increase obviously during the lag-exponential phase transition. Considering the increase of ethanol tolerance and trehalose during the lag-exponential phase transition, it can be inferred that maybe trehalose and other mechanisms are responsible for cells ethanol tolerance in that period, not the Tps1 protein, and that Tps1 protein might be not indispensable as a ‘moonlight protein’ in yeast cell to withstand ethanol stress before the stationary phase during the batch fermentation. In other words, Tps1 protein might be important for withstanding ethanol stress under higher content ethanol stress at late fermentation period as discussed before.

Interestingly, the expression of trehalose degraded genes (i.e., ATH1, NTH1, and NTH2) reached the maximal value at stationary phase and increased significantly than previous two phases ($P<0.05$) (Fig. 3). In spite of this, the trehalose content at stationary phase was still higher than that at lag phase probably due to the up-regulation of TPS1. As batch fermentation without exogenous feeding progressed to the stationary phase, the initial added nutrients including glucose were gradually depleted (Fig. S1C). Under such situation, lacking of energy and carbon source might make the degradation of trehalose to supply carbon source and energy for keeping cell survival necessary and induce the up-regulation of ATH1, NTH1, and NTH2 in despite of the continuously decreased pH value. In fact, previous studies also illustrated that trehalose is actually an important intracellular carbon source (Mansure et al. 1994; Van Dijck et al. 1995), and accumulated trehalose can be readily consumed to provide energy under carbon source-starved
situation through a mechanism implicating Ath1 protein (Jules et al. 2008). The slight decline of
trehalose content at stationary phase in comparison to that at exponential phase (Fig. 1) might also
illustrate the degradation of trehalose for the purpose of providing carbon source and energy at
stationary phase.

In summary, the trehalose accumulated in the bioethanol batch fermentation. Moreover,
during the lag phase-exponential phase transition, the accumulation of trehalose was not result
from the change of expression of trehalose synthetic genes but the down-regulation of NTH1 and
NTH2. In addition, an interesting hypothesis can be put forward that, at the transition of
lag-exponential phase, trehalose is responsible for the increase of ethanol tolerance; while, during
the exponential phase-stationary phase transition, the up-regulation of TPS1 contributed to the
accumulation of trehalose, Tps1 protein might be more important in yeast cell to withstand ethanol
stress at the stationary phase.

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Figure captions

**Fig. 1** Change of trehalose content during the bioethanol batch fermentation.

**Fig. 2** Changes of expressions of trehalose synthetic genes during the bioethanol batch fermentation. (A) *TPS1*. (B) *TPS2*. (C) *TPS3*. (D) *TSL1*.

**Fig. 3** Changes of expressions of trehalose degraded genes during the bioethanol batch fermentation. (A) *ATH1*. (B) *NTH1*. (C) *NTH2*.

**Fig. 4** Pair-wise correlations between trehalose content and trehalose synthetic genes during lag phase-exponential phase transition in scatter plots. (A) *TPS1*, $P=0.789$, $r=-0.141$. (B) *TPS2*, $P=0.118$, $r=-0.621$. (C) *TPS3*, $P=0.341$, $r=-0.475$. (D) *TSL1*, $P=0.730$, $r=0.182$.

**Fig. 5** Pair-wise correlations between trehalose content and trehalose degraded genes during lag phase-exponential phase transition in scatter plots. (A) *ATH1*, $P=0.384$, $r=-0.439$. (B) *NTH1*, $P=0.006$, $r=-0.935$. (C) *NTH2*, $P=0.001$, $r=-0.969$. 
### Table 1 Oligonucleotides used in this study

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<td>TPS2-F/R</td>
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Fig. 1

The graph shows the concentration of trehalose (mg/g dry weight) over time (2 h, 8 h, 16 h) with error bars representing the standard deviation. The statistical significance is indicated by the bars above the bars:

- From 2 h to 8 h, the difference is significant at P < 0.01.
- From 8 h to 16 h, the difference is not significant at P > 0.05.
- From 2 h to 16 h, the difference is significant at P < 0.01.
Fig. 2

A

B

C

D

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Fig. 4

A. Expression of TPS1 (fold change) vs. Trehalose (mg/g dry weight)

B. Expression of TPS2 (fold change) vs. Trehalose (mg/g dry weight)

C. Expression of TPS3 (fold change) vs. Trehalose (mg/g dry weight)

D. Expression of TSL1 (fold change) vs. Trehalose (mg/g dry weight)
Fig. 5

A: Expression of NTH1 (fold change) vs. Trehalose (mg/g dry weight)

B: Expression of NTH1 (fold change) vs. Trehalose (mg/g dry weight)

C: Expression of NTH2 (fold change) vs. Trehalose (mg/g dry weight)