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A Physiogenomic Study of the Tolerance of *Saccharomyces cerevisiae* to Isoamyl Alcohol

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Abstract: Isoamyl alcohol is a clear, unpleasantly odorous, colorless liquid of higher alcohol that emits a fruity aroma when heavily diluted. It has received much attention in recent years as a new fuel with a high energy density. Isoamyl alcohol can be produced industrially by microbial fermentation. Still, its toxicity to host cells has limited its potential for industrial production, and the molecular mechanism of its toxic effects has not yet been elucidated. In this study, RNA-Seq technology was used to analyze the transcripts of *Saccharomyces cerevisiae* under normal conditions and in the presence of isoamyl alcohol (0.5 g/L and 2.5 g/L). The results showed that the expression of the cell wall (*CCW12*, *BGL2*, *NCW2* and *SUN4*), cell membrane (*ELO1*, *ERG2*, *FAA1*, and *OPI3*), translation and other structural genes were significantly down-regulated. The expression of genes related to ATP biosynthesis, NADPH biosynthesis (*ZWF1*), and metal ion transport (*PMC1*) proteins were up-regulated. Strains with key genes knocked out were cultured without isoamyl alcohol. Combined results suggested that isoamyl alcohol may affect cell wall stability and cell membrane fluidity, and the expression of genes related to ion homeostasis and energy production may play a protective role against isoamyl alcohol stress. By maintaining cell wall stability/membrane fluidity under isoamyl alcohol pressure, improving certain ion homeostasis, and generating energy/NADPH, it is possible to overcome the toxicity of isoamyl alcohol in industrial fermentation processes to a certain extent.

Keywords: isoamyl alcohol; *Saccharomyces cerevisiae*; RNA-Seq; gene knockout; growth inhibition

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1. Introduction

Isoamyl alcohol is a colorless liquid with an unpleasant odor, which produces a pleasant fruity aroma when highly diluted [1]. Isoamyl alcohol, as a new type of fuel, has higher energy density and lower moisture absorption ability compared with ethanol and other traditional fuels. Along with other higher alcohols, isoamyl alcohol has received a lot of attention [2]. Isoamyl alcohol as an alcohol fuel itself has the advantages of cleanliness, renewable, higher energy density with ethanol, lower water solubility, better mixing with traditional fuels, better adaptability with traditional engines and more convenient transportation and storage, all of which indicate that isoamyl alcohol as a new energy fuel has good potential, the development of isoamyl alcohol as a new fuel will help to achieve energy diversification [3]. The majority of studies on higher alcohol biosynthesis are carried out in prokaryotic hosts, such as *Escherichia coli* (*E. coli*) [4]. However, *E. coli* is not a microorganism that spontaneously produces higher alcohols, and it is also not tolerant to higher alcohols, causing growth retardation at concentrations as low as 1% *v*/*v* [5].

In recent years, there have been increasing strategies to improve isoamyl alcohol production using *S. cerevisiae* as host cells [6], because *S. cerevisiae* itself produces isoamyl alcohol through the leucine degradation pathway (Figure S1) [3]. *S. cerevisiae* is widely used in the biotechnology industry and is generally considered safe due to its fully sequenced

genome and well-characterized metabolic pathway [7]. There are several ways to increase isoamyl alcohol production using *S. cerevisiae* as a host cell, the main approaches include using plasmid-based systems to overexpress pathway-specific genes [8], eliminating competing pathways and resolving cofactor imbalance [9]. An artificial protein scaffold was used to pull dihydroxy dehydrase and α-IPM synthetase to their closest proximity, and the results showed a more than 2-fold increase in isoamyl alcohol production, with the best producer yielding 522.76 ± 38.88 mg/L [10]. This yield was much higher than that of *E. coli* as host cells, but the final output is still far short of industrial production standards [4]. It is speculated that the reason for this result is the toxicity of isoamyl alcohol to *S. cerevisiae*.

Many previous studies have confirmed the inhibitory effect of alcohol on the growth and reproduction of *S. cerevisiae*. Among them, ethanol has been studied the most. Too high a concentration of ethanol will affect cell structure, mitochondrial enzyme activity, plasma membrane, and cell wall components, and eventually inhibit cell growth and even lead to cell death [11,12]. Similar results have been found for higher alcohols, toxicity being a key issue in the actual production of biobutanol [11]. Whether high concentration of isoamyl alcohol can have irreversible effects on the cell structure and physiological state of *S. cerevisiae* remains unclear, and no relevant literature has been published. Possibly because isoamyl alcohol was previously considered as a secondary metabolite of *S. cerevisiae* [10], its inhibitory effect on *S. cerevisiae* has not been studied. However, if *S. cerevisiae* is used to produce isoamyl alcohol, this problem cannot be circumvented.

In this study, *S. cerevisiae* was cultured under different concentrations of isoamyl alcohol stress to determine whether isoamyl alcohol has toxicity to *S. cerevisiae* and the magnitude of toxicit [13]. In addition, transcriptome analysis was used to determine the functional genes that play a key role under isoamyl alcohol pressure, and the knockout strains of related genes were further cultured and verified to prove whether these key genes play important roles under isoamyl alcohol stress.

2. Materials and Methods

2.1. Yeast Strains

The YKO library is also called "yeast knockout strains open biosystems", which are *S. cerevisiae* BY4741 with a genotype of *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*. Each single deletion mutant also contains a unique tag, "barcodes". For our screening experiment, we used the YKO *MATa* Strain Collection, which contains 5155 unique ORFs made in the BY4741 (*his3-Δ1 leu2-Δ0 ura3-Δ0 met15-Δ0*) background [14]. The knockout strains used in this experiment are shown in Table 1.

Table 1. List of *S. cerevisiae* strains used in this study and their genotypes.

YKO Lib	Strains	Background
BY4741	*BY4741, ccw12Δ, bgl2Δ, sun4Δ, elo1Δ, erg2Δ, opi3Δ, ncw2Δ, faa1Δ, ftr1Δ, fet3Δ, pmc1Δ, zwf1Δ, ino1Δ, lys12Δ, ade4Δ, ald6Δ, trm2Δ, fcy2Δ, eft1Δ, sul2Δ, izh2Δ, izh1Δ, btn2Δ, grx8Δ, pdc1Δ, met5Δ, pdc6Δ, gas1Δ, mae1Δ, jlp1Δ, bna2Δ, hmt1Δ, rps12Δ, spi1Δ, hsp150Δ, hsp26Δ, leu4Δ, leu9Δ, oac1Δ, zps1Δ, zno1Δ, utr2Δ, exg1Δ, scs7Δ, lac1Δ, zrt1Δ, are1Δ, vcx1Δ, ctr2Δ, nhx1Δ, mrs2Δ, gdt1Δ, tat1Δ, atx2Δ, hxt1Δ, gsf2Δ, tdh1Δ, alg8Δ, lpd1Δ, sdh1Δ, atg8Δ, mdh1Δ, sol3Δ, gnd1Δ.*	BY family

2.2. Media

YPD liquid and solid media: Bacto-yeast extract 1% (*w*/*v*), Bacto-peptone 2% (*w*/*v*), Glucose 2% (*w*/*v*), Geneticine (500 × G418, for knockout genes). Minimal synthetic media: Bacto-yeast nitrogen base w/o Amino acids, w/o Ammonium sulfate 0.17% (*w*/*v*), Ammonium sulphate 0.5% (*w*/*v*), Glucose 2% (*w*/*v*), and amino acid mixture.

2.3. Isoamyl Alcohol Tolerance Experiment

Preculture was obtained from a single colony that was inoculated into 20 mL YPD liquid medium and incubated overnight (16 h) at 30 °C, 200 rpm. After activation, the strains were cultured at 30 °C and 200 rpm in liquid YPD medium with different isoamyl alcohol concentrations of 0 g/L, 0.5 g/L, 1.5 g/L, 2.5 g/L, 3.5 g/Land 4.5 g/L. A spectrophotometer was used to monitor the growth at 600 nm every hour and calculate the specific growth rate. All experiments were set up in triplicate.

2.4. RNA-seq

Two different concentrations of isoamyl alcohol were used besides the control condition without isoamyl alcohol: (i) 0.5 g/L, assumed to be a 'non-inhibitory concentration' (ii) 2.5 g/L, assumed to be an 'inhibitory concentration'. The expression level of the gene was calculated by RSEM (v1.2.12), and differential expression analysis was performed using the DESeq2 (v1.4.5).

2.5. Genetic Targets Verification

Petri dishes containing YPD agar medium with an isoamyl alcohol concentration of 0 g/L, 0.5 g/L, 1.5 g/L, 2.5 g/L, 3.5 g/L, and 4.5 g/L were inoculated at initial OD600 of 1 and several dilutions. Cultures were carried out at 30 °C and observed and photographed every 4 h.

2.6. GO

Gene Ontology (GO) is an internationally standardized gene functional classification system that provides a dynamically updated controlled vocabulary to comprehensively describe the attributes of genes and gene products in living organisms. GO has a total of three ontologies, which describe the molecular function of genes, cellular components, and biological processes involved. The basic unit of GO is the term, and each term corresponds to an attribute.

Based on the GO annotation results and official classification, differentially expressed genes are functionally classified, and enrichment analysis is performed using the phyper function in R software to calculate pvalue. PValue is then corrected for FDR and functions with Q value ≤ 0.05 are typically considered significantly enriched. Enriching up-regulated genes and down-regulated genes separately to obtain relevant GOterms, and analyzing the ones with a higher number of gene enrichment.

3. Results and Discussion

3.1. Tolerance Test

The hourly absorbance of *S. cerevisiae* BY4741 in YPD liquid medium with different concentrations of isoamyl alcohol is shown in Table S1. The growth curves of WT BY4741 under different concentrations of Isoamyl alcohol are shown in Figure S2. with the specific growth rate (μ) under different concentrations determined. The specific growth rate of *S. cerevisiae* under different isoamyl alcohol concentrations was plotted as Figure 1a. It can be seen that the growth of *S. cerevisiae* under the pressure of isoamyl alcohol was significantly inhibited. When the isoamyl alcohol concentration reached 0.5 g/L, the specific growth rate was 97% of that at 0 g/L; at an isoamyl alcohol concentration of 2.5 g/L, the specific growth rate was 74.8% of that at 0 g/L; when the isoamyl alcohol concentration was 4.5 g/L, the growth rate of *S. cerevisiae* was 50% lower.

By observing the growth state of BY4741 in the solid medium in Figure 1b, we found that the inhibition effect was more obvious at the concentration of 2.5 g/L and almost no colony growth at the concentration of 5 g/L. We considered that there was sufficient nutrition in a liquid medium, and the yeast did not show sensitivity to isoamyl alcohol in a short time, so in the follow-up experiment, we selected three isoamyl alcohol concentration gradients of 0 g/L, 0.5 g/L, and 2.5 g/L for transcriptome analysis.

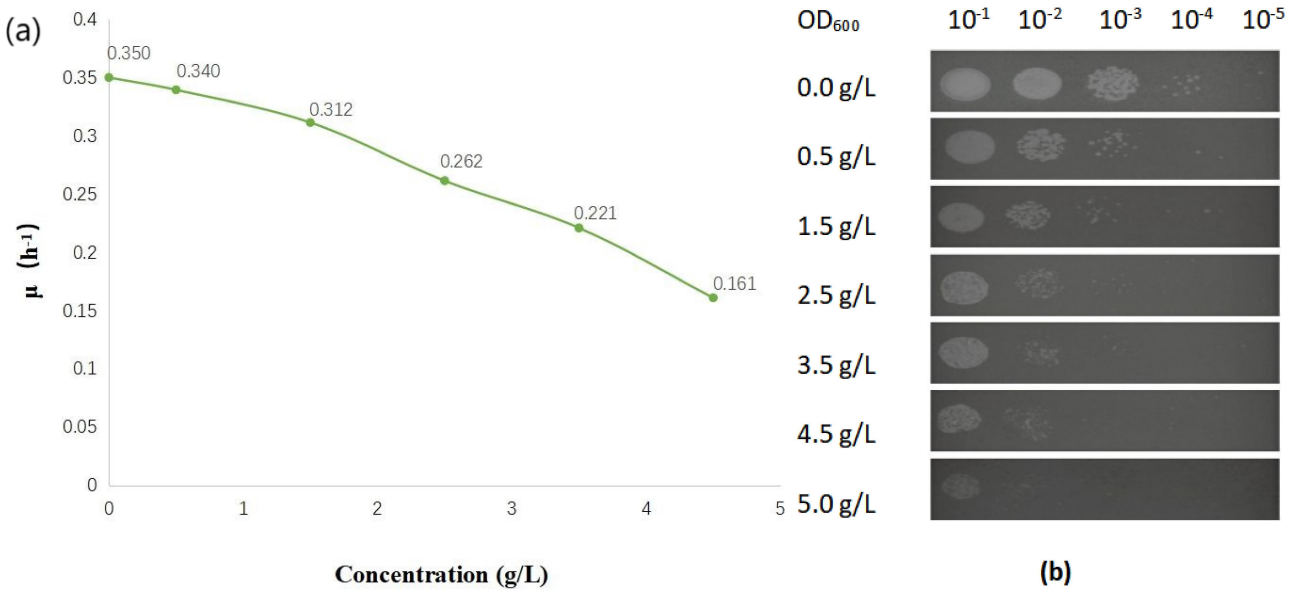

Figure 1. (**a**) The growth rate (μ) of *S. cerevisiae* BY4741 under 0 g/L, 0.5 g/L, 1.5 g/L, 2.5 g/L, 3.5 g/L, 4.5 g/L of isoamyl alcohol. (**b**) Growth status of BY4741 on solid medium containing different concentrations of isoamyl alcohol at 36 h.

3.2. Transcriptome Analysis

The repression of isoamyl alcohol on the growth and metabolism of *S. cerevisiae* is the bottleneck of industrial production of microbial fermentation. Functional enrichment analysis of differentially expressed genes in *S. cerevisiae* under isoamyl alcohol stress was performed by RNA-Seq (Table S2), and the GO terms found, then GO enrichment analysis was analyzed (Figure 2). In light of these results, we have selected several terms that we believe to be important for the vital activity of S. cerevisiae, such as cell wall, cell membrane, metal ion transport, vesicles, and mitochondria, for a further in-depth discussion.

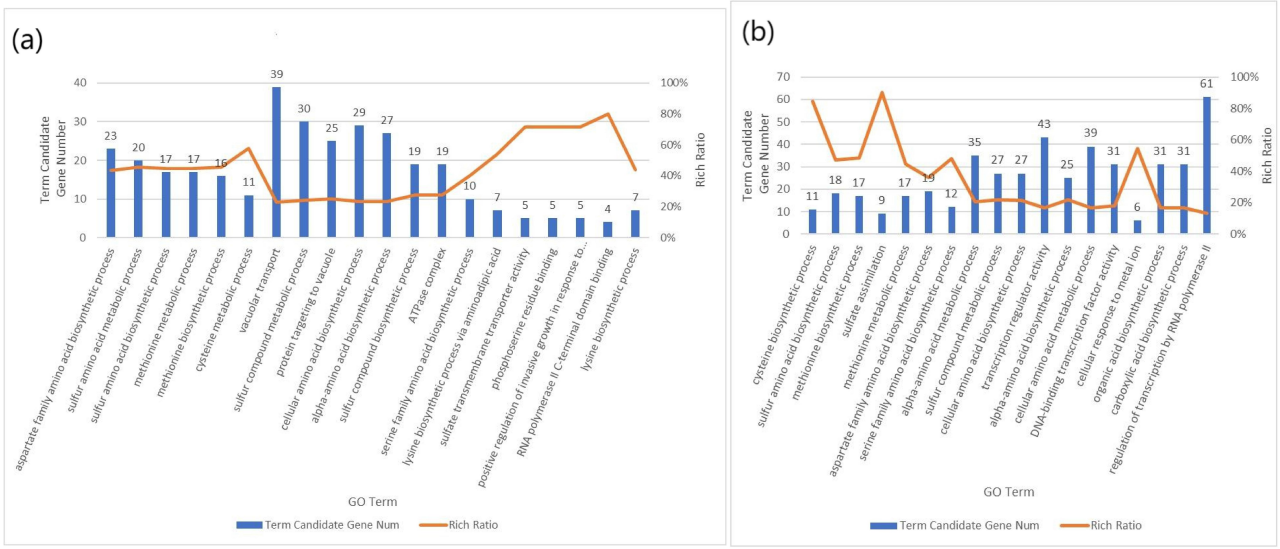

Figure 2. *Cont.*

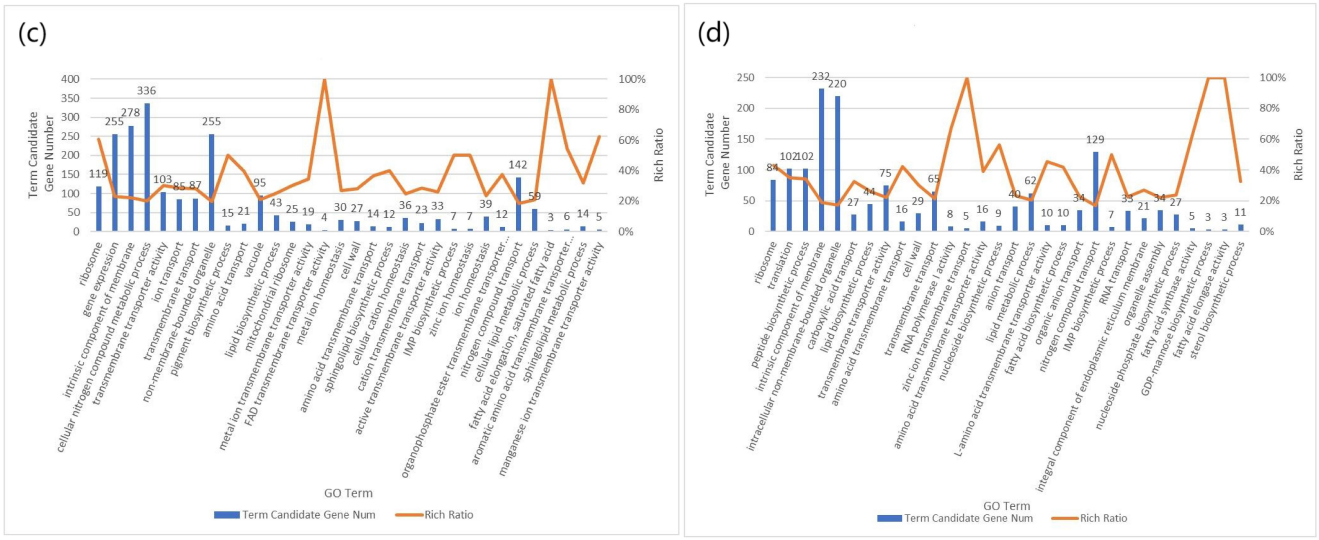

Figure 2. Figure of GO enrichment analysis. (**a**) GO enrichment analysis of BY4741 up-regulated differential gene at 0 g/L and 0.5 g/L isoamyl alcohol concentrations. (**b**) enrichment analysis of BY4741 up-regulated differential gene at 0 g/L and 2.5 g/L isoamyl alcohol concentration. (**c**) enrichment analysis of BY4741 down-regulated differential gene at 0 g/L and 0.5 g/L isoamyl alcohol concentrations. (**d**) GO enrichment analysis of BY4741 down-regulated differential gene at 0 g/L and 2.5 g/L isoamyl alcohol concentrations.

3.3. Cell Wall

The *S. cerevisiae* cell wall accounts for approximately 15–30% of the dry weight. The inner layer is mainly composed of *β*-1,3-glucan chain (80–90%), *β*-1,6-glucan chain (8–18%) and a small amount of chitin chain (1–2%) [15]. Due to the helical nature of the *β*-1,3-glucan chain, this layer is primarily responsible for the mechanical strength and elasticity of the cell wall [16]. As shown in Table 2, the genes with significant differences in mannose and glucan GO enrichment were down-regulated genes. Moreover, the down-regulated functional genes are mainly related to biosynthesis, such as *BGL2*, *FKS1*, *EXG1*, which are all *β*-1,3-glucan biosynthetase regulatory genes. However, the expression of some genes related to chitin is up-regulated, and the blocked expression of *BGL2* gene also will lead to increased chitin content in the cell wall. Therefore, we speculated under isoamyl alcohol pressure in the cell wall that the amount of mannose and glucan decreased and the amount of chitin increased, leading to a weakening in the mechanical strength and protection of the cell wall.

As shown in Table 2, the genes involved in mannose-related structural components of the cell wall with high and significant differences in expression are *CIS3*, *SRL1*, *TIP1*, *CCW12*, and same as genes related to chitin (*UTR2*), glucan (*EXG1*, *KNH1*, *SUN4*, *BGL2*, *SCW10*), All of these genes were down-regulated under isoamyl alcohol stress, and it is assumed that cell wall-related genes were inhibited as sensitive genes under isoamyl alcohol stress, leading to chemical changes in the cell wall structure and impaired biogenesis and maintenance mechanisms, thus affecting the permeability of the cell wall and further affecting yeast cell growth and reproduction.

Among the glucan-related enriched genes, we found an interesting gene, *NCW2*. Previous studies have shown that *NCW2*, a functional gene involved in cell wall integrity (CWI) regulon [17] participate in cell wall repair, is usually up-regulated when the cell wall is stimulated [18]. In most cases, *NCW2* is up-regulated when the cell wall is stimulated by external stimuli, such as when yeast cells are exposed to PHMB, the gene shows a 7-fold up-regulation growth; it also shows slight up-regulation under high concentration ethanol stress; osmotic stress, heat stress, and acid stress do not alter the expression of *NCW2* gene, while gene expression is continuously down-regulated under isoamyl stress. However, under isoamyl alcohol stress, the expression of *NCW2* was continuously down-regulated.

The key functional genes in the CWI signaling pathway such as *SLT2*, *RLM1*, *ROM2*, *RHO1*, *PKC1*, *BCK1*, *MKK1*, and *SLT2* were up-regulated under isoamyl alcohol stress [19]. We predicted that the signaling pathway of CWI was still activated under isoamyl alcohol stress, but the expression of *NCW2* was specifically inhibited by isoamyl alcohol, failing to synthesize or assemble the substances that repair cell wall structure and endow cell rigidity.

Table 2. Differential expression of cell wall related genes in transcriptome analysis.

Go-Terms	Difference Multiple Value Range	In Range from Cluster *
Mannose	$\log_2 \frac{0.5g/L}{0g/L} < -1$	***CCW12***, ***SRL1***, ***TIP1***, ***MNN2***, *PMT1*, ***MNN11***, ***PMT4***, ***KTR1***
	$\log_2 \frac{2.5g/L}{0g/L} < -1$	***CCW12***, ***SRL1***, ***TIP1***, *CIS3*, ***MNN2***, ***MNN11***, ***PMT4***, ***KTR1***
	$\log_2 \frac{2.5g/L}{0.5g/L} < -1$	***CCW12***, ***SRL1***, ***KTR1***
Chitin	$\log_2 \frac{0.5g/L}{0g/L} < -1$	***UTR2***, ***BGL2***, *CHS3*, *PCM1*
	$\log_2 \frac{2.5g/L}{0g/L} < -1$	***UTR2***, ***BGL2***
	$\log_2 \frac{2.5g/L}{0g/L} > -1$	*CRZ1*, *CHS1*
Glucan	$\log_2 \frac{0.5g/L}{0g/L} < -1$	***BGL2***, ***SUN4***, ***KNH1***, ***EXG1***, ***FKS1***, ***SCW10***, *NCW2*
	$\log_2 \frac{2.5g/L}{0g/L} < -1$	***BGL2***, ***SUN4***, ***KNH1***, ***EXG1***, ***FKS1***, ***SCW10***

* The following bold genes show the same trend of change under different concentrations of comparison.

Based on the above analysis, we selected four potential genes (*CCW12*, *BGL2*, *SUN4*, *NCW2*) that may be strongly affected by isoamyl alcohol, further study about strain *ccw12*Δ, *bgl2*Δ, *sun4*Δ, *ncw2*Δ under isoamyl alcohol stress was carried out.

3.4. Cell Membrane

Ethanol can disrupt proton homeostasis by increasing membrane fluidity, reducing membrane integrity, and increasing ion permeability. Similar to ethanol, 1-butanol affects membrane lipid composition and nutrient transport in addition to inhibiting the initiation of translation. By demonstrating that the addition of a certain amount of fatty acids, particularly unsaturated fatty acids, to the culture medium, which can be taken up by yeast cells and fused into the cell and mitochondrial membranes, it was found that these yeast cells showed a significant increase in ethanol tolerance during ethanol fermentation, confirming that fatty acids can counteract the fluidizing effect of ethanol on the cell membrane. As a model organism with high ethanol tolerance, *S. cerevisiae* cells themselves can synthesize more long-chain unsaturated fatty acids during growth compared to other microorganisms, resulting in increased cellular ethanol tolerance. However, in our experiment, genes related to fatty acid synthesis in cell membranes were generally down-regulated under isoamyl alcohol conditions, with more obvious differences in expression including *OLE1, TSC13, ELO1, ELO2*, and *ELO3*. Similarly, the addition of ergosterol to the medium not only promoted yeast growth and increased ethanol production but also increased ethanol tolerance when the cell membrane of *S. cerevisiae* was rich in ergosterol or soy sterols than when it was rich in rape sterols or cholesterol, and the higher the ergosterol content, the more ethanol tolerant the organism was. It is assumed that ergosterol also increases the toughness of cell membranes and reduces membrane fluidity [20,21]. In our experimental results, GO cluster analysis showed that the main genes, such as *ERG2, ARE1, CYB5* related to ergosterol biosynthesis were down-regulated. Therefore according to transcriptome analysis in Table 3, due to the two components that can enhance cell membrane fluidity, fatty acids, and ergosterol, their biosynthesis-related genes are down-regulated under isoamyl alcohol pressure, which may lead to a further increase in membrane fluidity. Among them, *ELO1* and *ERG2* were the most down-regulated genes. Interestingly, one gene related to long chain fatty acyl-CoA synthetase, *FAA1*, was not affected, and its expression level was continuously up-regulated with the increase in isoamyl alcohol concentration [22]. We speculate that *S. cerevisiae* cells can also utilize exogenous fatty acids activated by

Faa1p when the expression of fatty acid synthetase complex is down-regulated by isoamyl alcohol inhibitors.

Phospholipids constitute most of the lipids in bilayer membranes, and therefore, they play an important role in determining the physical and chemical properties of membranes [23]. As shown in Table 3, the number of significantly different genes related to phospholipids was higher, so these genes were classified in more detail. For the up-regulated differential genes, GO classification includes phosphatidylinositol binding, transport, and CVT pathways. In the down-regulated differential genes, the GO classification mainly includes integral to membrane, transferase activity, and phospholipid biosynthesis. Among them, due to speculation *SLC1, SCS2, SCS3, OPI3*, and *PIS1* genes related to phospholipid biosynthesis to play an important role in the influence of cell membrane function, and *OPI3* has the highest expression level.

Table 3. Differential expression of membrane related genes in transcriptome analysis.

Go-Terms	Difference Multiple Value Range	In Range from Cluster *
Fatty acid	$\log_2 \frac{0.5g/L}{0g/L} < -1$	***ELO1, YAT1, ELO2, TSC13, ACP1, ELO3, IZH2, ERG10, AIM45, OLE1,*** *FAT1, OAR1, FAA4, SCS7*
	$\log_2 \frac{0.5g/L}{0g/L} > 1$	***RTG1, FAA1,*** *IDP3*
	$\log_2 \frac{2.5g/L}{0g/L} < -1$	***YAT1, ELO2, TSC13, OLE1, ELO1, ACP1, ELO3, IZH2, ERG10, AIM45***
	$\log_2 \frac{2.5g/L}{0g/L} > 1$	***RTG1, FAA1***
Ergosterol	$\log_2 \frac{0.5g/L}{0g/L} < -1$	***ERG4, ERG25, NCP1, ERG3, HMG1, ERG13, ERG2, ERG10, CYB5,*** *ARE1, ERG11*
	$\log_2 \frac{0.5g/L}{0g/L} > 1$	***GRE2,*** *ARE2*
	$\log_2 \frac{2.5g/L}{0g/L} < -1$	***ERG4, ERG25, NCP1, ERG3, HMG1, ERG13, ERG2, ERG10, CYB5***
	$\log_2 \frac{2.5g/L}{0g/L} > 1$	***GRE2***
Phospholipid	$\log_2 \frac{0.5g/L}{0g/L} < -1$	***ALG7, PER1, SLC1, GPI11, SPF1, SCS2, SCS3, GPI10, EGD2, OPI3, AUR1, ARV1, GAB1, HMG1, ERG13, PLB2, ANY1, CDC33, ARF3, PIS1,*** *IPT1, AGE1, ATG27, SEC59, SCS7, OPT2*
	$\log_2 \frac{0.5g/L}{0g/L} > 1$	***SWH1, ATG8, ATG20, IVY1, VAM7, BBC1, PDR16, MET22, VPS30,*** *INO1*
	$\log_2 \frac{2.5g/L}{0g/L} < -1$	***ALG7, PER1, SLC1, GPI11, SPF1, SCS2, SCS3, GPI10, EGD2, OPI3, AUR1, ARV1, GAB1, HMG1, ERG13, PLB2, ANY1, CDC33, ARF3, PIS1***
	$\log_2 \frac{2.5g/L}{0g/L} > 1$	***SWH1, ATG8, ATG20, IVY1, VAM7, BBC1, PDR16, MET22, VPS30***

* The following bold genes show the same trend of change under different concentrations of comparison.

Therefore, we selected four potential genes (*ELO1, FAA1, ERG2, OPI3*) that may be strongly affected by isoamyl alcohol, further study about strain *opi3*Δ, *elo1*Δ, *erg2*Δ, *faa1*Δ under isoamyl alcohol stress was carried out.

3.5. Metal Ion Transporter

Table 4 shows that, in the GO enrichment results, all the significantly differentially expressed genes of Calcium ion transport, except *PMC1*, were down-regulated. It can be seen that *PMC1* plays a key role under isoamyl alcohol pressure. *PMC1* is known to be located in the fungi-type tonoplast membrane and is involved in the regulation of calcium homeostasis through transmembrane transport [24,25]. We predict that the intracellular ion balance is disrupted under isoamyl alcohol stress and further study about strain *pmc1*Δ under isoamyl alcohol stress was carried out.

Table 4. Differential expression of metal ion and vacuolar related genes in transcriptome analysis.

Go-Terms	Difference Multiple Value Range	In Range from Cluster *
Calcium ion transport	$\log_2 \frac{0.5g/L}{0g/L} < -1$	***FTR1, FET3, SIT1,*** *FLC2*
	$\log_2 \frac{0.5g/L}{0g/L} > 1$	***PMC1***
	$\log_2 \frac{2.5g/L}{0g/L} < -1$	***FLC2, GDT1, VCX1, SPF1***
	$\log_2 \frac{2.5g/L}{0g/L} > 1$	***PMC1***
Vacuolar homeostasis	$\log_2 \frac{0.5g/L}{0g/L} < -1$	***GDT1, PER1, VCX1, SIT1, VMA16, ZRT3, EMP70, PFK2, ZRC1, CTR2,*** *TMN2, NHX1, VMA3, TMN3, FET5, SMF2, YHC3, VNX1, ENB1*
	$\log_2 \frac{0.5g/L}{0g/L} > 1$	***PMC1, VPS52, VMA22, GEX1,*** *VPS3*
	$\log_2 \frac{2.5g/L}{0g/L} < -1$	***GDT1, PER1, VCX1, SIT1, VMA16, CTR2, ZRT3, EMP70, PFK2, ZRC1***
	$\log_2 \frac{2.5g/L}{0g/L} > 1$	***PMC1, VPS52, VMA22, GEX1,*** *MNR2*

* The following bold genes show the same trend of change under different concentrations of comparison.

3.6. Mitochondrial Activity

Mitochondria are the main organelles in cells that produce energy. A large number of mitochondria-related differential genes were obtained through GO enrichment, and further, GO classification of these genes found that the function of both up-regulated and down-regulated genes was related to ATP. In the ATP biosynthetic process of mitochondria, ATP synthase and the electron transport chain play an important role [26–29], especially the genes associated with ATP synthase and the electron transport chain. The expression of most genes was up-regulated, which suggested that more ATP was needed to satisfy the life activities of yeast cells under isoamyl alcohol stress. However, only *CYC1* was down-regulated with increasing isoamyl alcohol concentration.

CYC1 is known to encode the electron carrier protein cytochrome c. Cytochrome c facilitates the transfer of electrons from respiratory complex III to respiratory complex IV via its covalently linked hemoglobin moiety [30]. In *S. cerevisiae* cells, which account for 95% of total cytochrome c, down-regulation of*CYC1* expression is likely to result in inhibition of *S. cerevisiae* growth [31]. Previous studies have shown that *CYC1* expression is usually induced or repressed directly at the promoter transcription rather than at the translational stage and that the *CYC1* activation sequence *UAS2* represses *CYC1* transcription by binding to Mig1p and ccaat binding activation complex subunits Hap2p and Hap3p. The expression levels of *MIG1*, *HAP2*, and *HAP3* were all up-regulated, and it was hypothesized that isoamyl alcohol inhibited the expression of *CYC1* by stimulating the expression of *MIG1*.

3.7. Pentose Phosphate Pathway

The pentose phosphate pathway (PPP) is also known as hexose monophosphate shunt. Large amounts of NADPH are produced by reducing the coenzyme $NADP^+$ [32]. NADPH provides reducing agents for a variety of synthetic reactions within cells and is involved in several anabolic reactions, such as the synthesis of lipids, fatty acids, and nucleotides. This pathway also provides reducing energy for various enzymes involved in stress response [33]. Although no PPP and NADPH-related terms were found in GO enrichment analysis, this may be due to the limited number of enzyme regulatory genes that catalyze this process. *ZWF1, SOL3/SOL4* and *GND1/GND2* are the enzymes encoding the reactions that catalyze NADPH production and constitute the first [34], second and third steps of PPP, respectively. Based on differential gene analysis, these genes showed up-regulation. This may suggest that yeast cells require more NADPH to provide reducing energy for the enzymes required for various stress responses. To verify the importance of NADPH for resistance to isoamyl alcohol, Strain *zwf1*Δ under isoamyl alcohol stress was further analyzed.

3.8. Further Study on Potential Functional Genes

Based on the above analysis, we screened out several potential genes that may play an important role in yeast cell growth under isoamyl alcohol stress. These include *NCW2*, *FAA1*, *PMC1*, *ZWF1*, which may play a special molecular mechanism under isoamyl alcohol stress. It also includes genes that are highly expressed and representative in each functional classification, such as *CCW12*, *BGL2* and *SUN4* in the cell wall and *OPI3*, *ELO1* and *ERG2* in the cell membrane. All of the above potential functional genes were further analyzed.

According to the Figure 3, the growth state of *ncw2*Δ,*ccw12*Δ, *bgl2*Δ, *elo1*Δ, *erg2*Δ, *faa1*Δ, *zwf1*Δ strains were significantly slower than that of wild type BY4741. The growth of *ncw2*Δ, *ccw12*Δ and *bgl2*Δ strains were severely inhibited under the condition of 0 g/L isoamyl alcohol. It is speculated that *CCW12* and *BGL2*, as regulatory genes of mannoglycoprotein on the cell wall, play an important role in the maintenance of new synthetic regions in the cell wall. Similarly, *NCW2*, as a gene involved in cell wall repair, determines whether the cell wall can continue to function despite damage. These results show that *NCW2*, *CCW12*, and *BGL2* play important roles in the maintenance of life activities of *S. cerevisiae* under normal conditions. Under isoamyl alcohol, the downregulation of *CCW12*, *BGL2* further impaired the life activities of *S. cerevisiae*.

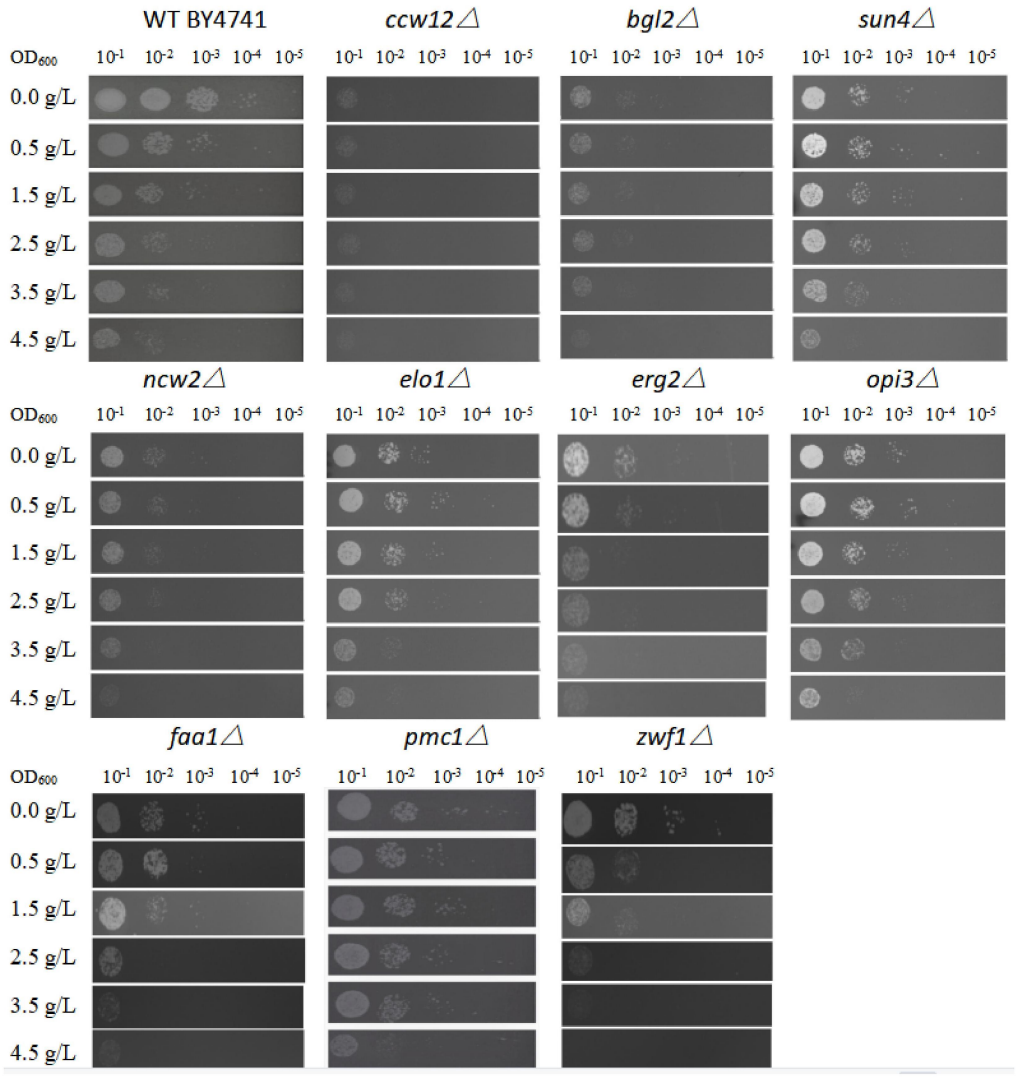

Figure 3. Growth state of WT BY4741 and gene deletion yeast in solid medium with different concentrations of isoamyl alcohol at 36 h.

The *erg2*Δ, *faa1*Δ and *zwf1*Δ strains can still grow normally at 0 g/L, but with the increase in isoamyl alcohol concentration, they show more sensitivity to isoamyl alcohol than WT. It can be seen from Figure 4a that with the increase in isoamyl alcohol concentration, the specific growth rate of *elo1*Δ is the same as that of WT BY4741, while *erg2*Δ shows a higher sensitivity when the isoamyl alcohol concentration reaches above 0.5 g/L, and completely stops growth at 4.5 g/L isoamyl alcohol concentration. The results showed that *ELO1* deletion did not make the strain more sensitive to isoamyl alcohol. As an isomerase regulatory gene, *ERG2* is mainly used to promote the biosynthesis of ergosterol, and its gene deletion strains show high sensitivity to isoamyl alcohol. This proves that ergosterol plays an important role under isoamyl alcohol pressure. The gene expressions of *FAA1*, *PMC1*, and *ZWF1* were up-regulated in transcriptome analysis. Combined with Figures 3 and 4b, we found that *faa1*Δ, *pmc1*Δ and *zwf1*Δ strains grew well in normal YPD medium but exhibited much higher sensitivity than WT BY4741 in the presence of isoamyl alcohols, which proved that the functional expression of genes *FAA1*, *PMC1*, and *ZWF1* played a role in resistance to isoamyl alcohol. Earlier in this paper, we described the functions of genes *FAA1*, *PMC1*, and *ZWF1* and speculated the molecular mechanism of resistance to isoamyl alcohol stress after the expression of these two genes. The culture results of the gene deletion strain are consistent with our hypothesis.

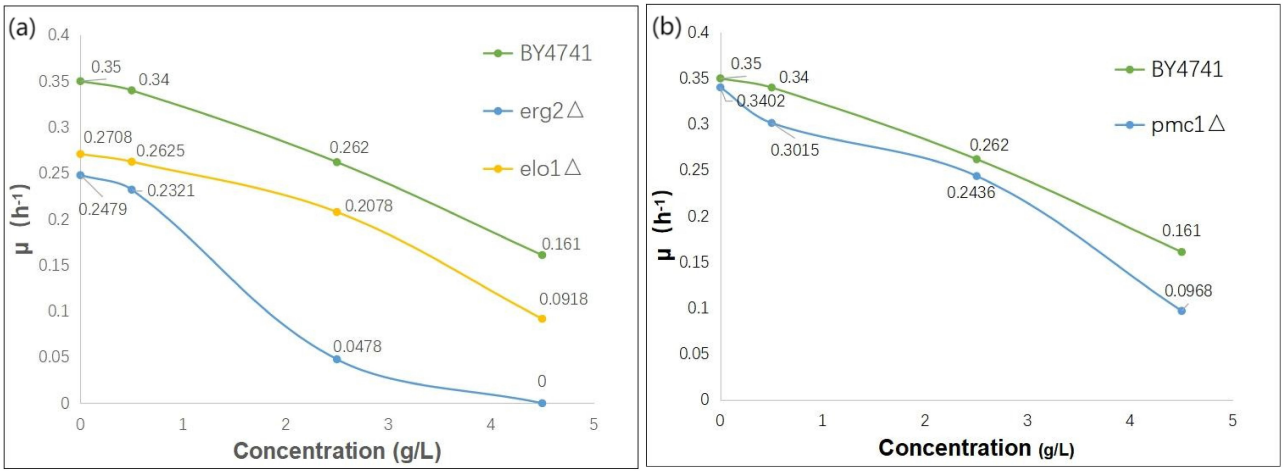

Figure 4. Specific growth rate of cell (**a**) *erg2*Δ, *elo1*Δ, (**b**) *pmc1*Δ in liquid medium under isoamyl alcohol conditions of 0 g/L, 0.5 g/L, 2.5 g/L, 4.5 g/L (μL).

The growth state of *sun4*Δ and *opi3*Δ strains in 0 g/L isoamyl alcohol was also decreased compared with that of wild type BY4741. However as the concentration of isoamyl alcohol increased, the strains did not show much sensitivity. *sun4*Δ showed no obvious growth inhibition before isoamyl alcohol concentration of 3.5 g/L. It is known that *sun4*Δ can cause low efficiency of mother-daughter cell separation, so it is speculated that *S. cerevisiae* may be more resistant to isoamyl alcohol in the pseudomycelium state. Gene *OPI3* controls the biosynthesis of phosphatidylcholine, which is an important component of biofilm, and its deletion affects the life activities of yeast. Combined with the growth status analysis of *opi3*Δ on solid medium, it was found that the deletion of phosphatidylcholine seemed to enhance the resistance of *S. cerevisiae* to isoamyl alcohol stress. This may be related to the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE), with the decrease in PC content and the increase in PE content in order to reduce the fluidity of the membrane and enhance the stability of the membrane.

4. Conclusions and Perspectives

To improve the production of isoamyl alcohol from *S. cerevisiae*, it is important to study the toxic effect of isoamyl alcohol on host cells. By exploring the molecular mechanism,

a feasible modification method was proposed to improve the tolerance of *S. cerevisiae* to isoamyl alcohol.

This study first verified the inhibitory effect of isoamyl alcohol on *S. cerevisiae* and transcriptome analysis of *S. cerevisiae* under isoamyl alcohol stress was performed using an RNA-Seq approach. To the authors' knowledge in the field, this study is the first systematic study of the molecular mechanisms underlying the changes in gene expression that occur in *S. cerevisiae* under isoamyl alcohol stress. Through GO enrichment tests, we have selected terms important for cellular life and analyzed their expression in detail to identify potential genes that may play important functions under isoamyl alcohol stress.

Under isoamyl alcohol pressure, the proportion of other substances, except chitin, in the cell wall decreased. These phenomena demonstrate that the cell wall does undergo deformation under isoamyl alcohol conditions, which may be related to stimulated by isoamyl alcohol the production of pseudo mycelium in *S. cerevisiae*. Upon stimulation with isoamyl alcohol, membrane stability is reduced and fluidity is increased, leading to an imbalance in intracellular ion homeostasis. Ion transport proteins in the vesicles play a role in stabilizing ion homeostasis, and we have observed that a large proportion of the ion transport process is mediated by metal ion transport proteins. The overall up-regulation of key gene expression related to ATP release in mitochondria. The expression of most genes related to ATP synthesis is up-regulated, and the regulatory genes required for the composition of ATP synthase components are overall up-regulated. The expression of enzymes related to NADPH synthesis steps in the PPP pathway is also up-regulated. Therefore, it is speculated that it may require more ATP and NADPH to maintain life activities under isopentyl stress. The key gene *FAA1*, which acts on the synthesis of fatty acids in the cell membrane, is up-regulated under the stimulation of isoamyl alcohol; therefore, it is speculated that it may be an important potential gene for resisting the inhibition of isoamyl alcohol. The ion transport proteins in vacuoles play an important role in stabilizing ion homeostasis. The absence of gene *PMC1* under conditions without isoamyl alcohol stimulation will not have a significant impact on the life activities of brewing yeast. However, under isoamyl alcohol stress, *PMC1* up-regulation expression can activate the activity of p-type calcium transporters, regulate more ion transporters to play a role, and thus maintain intracellular ion balance.

Until now, the lack of understanding of the mechanisms of inhibition of isoamyl alcohol in *S. cerevisiae* has kept the production of isoamyl alcohol through the biological fermentation route at a theoretical stage. In this experiment, some of the key genes like *NCW2, FAA1, SUN4, ERG2, PMC1, ZWF1* and the molecular mechanisms regulated by these genes were identified. This provides an essential theoretical basis for the study of the toxicity of isoamyl alcohol on *S. cerevisiae* and enriches the research system of the microbial cellular stress response. It provides new ideas for the development of highly tolerant strains and a modern theoretical basis for the biosynthetic pathway of *S. cerevisiae* as a host to enhance isoamyl alcohol production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10010004/s1, Table S1: OD value of WT By4741 under different concentrations of isoamyl alcohol; Table S2: RNA-seq analysis of wild-type BY4741 strains grown with or without isoamyl alcohol; Figure S1: *Saccharomyces cerevisiae* produces isoamyl alcohol through the leucine degradation pathway; Figure S2: Growth curve of WT By4741 under different concentrations of isoamyl alcohol.

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