

# Improving isobutanol productivity through adaptive laboratory evolution in *Saccharomyces cerevisiae*

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## Research

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# Abstract

**Background:** Isobutanol is an ideal second-generation biofuels due to its lower hygroscopicity, higher energy density and higher-octane value. However, isobutanol is toxic to production organisms. To improve isobutanol productivity, adaptive laboratory evolution method was carried out to improve the tolerance of *Saccharomyces cerevisiae* toward higher isobutanol and higher glucose concentration.

**Results:** We evolved the laboratory strain of *S. cerevisiae* W303-1A by using EMS (ethyl methanesulfonate) mutagenesis followed by adaptive laboratory evolution. The evolved strain EMS39 with significant increase in growth rate and viability in media with higher isobutanol and higher glucose concentration was obtained. Then, metabolic engineering of the evolved strain EMS39 as a platform for isobutanol production were carried out. Delta integration method was used to over-express *ILV3* gene and 2 $\mu$  plasmids carrying *ILV2*, *ILV5* and *ARO10* were used to over-express *ILV2*, *ILV5* and *ARO10* genes in the evolved strain EMS39 and wild type W303-1A. And the resulting strains was designated as strain EMS39V2 $\delta$ V3V5A10 and strain W303-1AV2 $\delta$ V3V5A10, respectively. Our results shown that isobutanol titers of the evolved strain EMS39 increased by 30% compared to the control strain. And isobutanol productivity of strain EMS39V2 $\delta$ V3V5A10 increased by 32.4% compared to strain W303-1AV2 $\delta$ V3V5A10. Whole genome resequencing and analysis of site-directed mutagenesis of the evolved strain EMS39 have identified important mutations. In addition, RNA-Seq-based transcriptomic analysis revealed cellular transcription profile changes resulting from EMS39.

**Conclusions:** With the aim of increase productivity of isobutanol in *S. cerevisiae*, improving tolerance toward higher isobutanol and higher glucose concentration via EMS mutagenesis followed by adaptive evolutionary engineering was conducted. An evolved strain EMS39 with significant increase in growth rate and viability had been obtained. And metabolic engineering of the evolved strain as a platform for isobutanol production was carried out. Furthermore, analysis of whole genome resequencing and transcriptome sequencing were also carried out.

## Background

Isobutanol has received more attentions as one of second generation biofuels that are compatible with current infrastructure due to their energy density and lower moisture absorption [1–15]. Budding yeast *Saccharomyces cerevisiae* can produce low amount of isobutanol via valine synthesis pathway [16–18]. And to improve isobutanol titers from glucose, researchers had performed many different strategies, such as over-expressing genes of valine biosynthesis or its degradation[2, 5], eliminating competing pathways[7, 9, 19–20] and resolving the cofactor imbalance [7], compartmentalizing the Ehrlich pathway into mitochondria [21] or re-locating the mitochondrial valine biosynthesis enzymes *Ilv2*, *Ilv5* and *Ilv3* into the cytosol via truncation of their mitochondrial targeting sequences[3]. While the productivity of the present engineered yeast strains is too low for industrial production. One of the problems that restrict isobutanol productivity is the tolerance toward both higher glucose and isobutanol concentration in *S. cerevisiae*.

Tolerance toward both higher glucose and isobutanol concentration does not seem to be a monogenic trait. As a complex phenotype, the evolution of such complex traits requires synergistic actions of many genes that are widely distributed throughout the genome. To circumvent this limitation, we enhance tolerance of *S. cerevisiae* to higher glucose and isobutanol concentration by adaptive laboratory evolution. Adaptive laboratory evolution was served as a genome-wide method for improving desirable phenotypes without the knowledge of genetic determinants for network information about those phenotypes[22–23]. And it was successfully employed to identify biological solutions to biofuel and alcohol toxicity in *S. cerevisiae* [24–30]. In this study, we used adaptive laboratory evolution to enhance yeast strain's tolerance toward higher glucose and isobutanol concentration in fermentation. It was based on a EMS (ethyl methanesulfonate) mutagenized W303-1A. After mutagenesis, strains were subjected to a 15 days stringent selection. By using this approach, we isolated one evolved strain EMS39 with significant increased growth rate and viability in fermentation.

Metabolic engineering of the evolved strain EMS39 as a platform for isobutanol production were carried out. 2  $\mu$  plasmids carrying ILV2, ILV5 and ARO10 were used to over-express ILV2, ILV5 and ARO10 genes in the evolved strain EMS39 and wild type strain W303-1A. It was reported that the enzyme dihydroxyacid dehydratase (encoded by ILV3) might be limiting the isobutanol pathway [11]. To break through this restriction point, we used  $\delta$ -integration method to increase the integrated copy number of ILV3 in EMS39 and W303-1A. It was reported that  $\delta$ -integration method could be used to over-express genes in yeast strains[31–33], Then, fermentation characters of strains EMS39V2 $\delta$ V3V5A10 and W303-1AV2 $\delta$ V3V5A10 were investigated. In addition, analysis of whole genome resequencing and transcriptome sequencing were also carried out to identify important mutations and significant changes in transcriptional levels that may caused higher isobutanol tolerance and higher isobutanol titers.

In summary, to increase productivity of isobutanol in *S. cerevisiae*, improving isobutanol tolerance and higher glucose concentration via EMS mutagenesis followed by adaptive evolutionary engineering was conducted. Evolved strain with significant increase in growth rate and viability has been obtained. Metabolic engineering of the evolved strain as a platform for isobutanol production revealed advantages of the evolved strain for micro-aerobic production of isobutanol. Whole genome re-sequencing and analysis of site-directed mutagenesis of the evolved strain have identified important mutations that has caused improved in growth rate and viability in *S. cerevisiae*. RNA-based transcriptomic analysis were also carried out to identify transcriptome perturbations that may have caused higher isobutanol titers in strain EMS39V2 $\delta$ V3V5A10.

## Results

### **EMS and adaptive evolutionary engineering improved micro-aerobic growth and isobutanol tolerance of *S.cerevisiae***

Our strain development strategy is outlined in Fig.1. We used methods of adaptive laboratory evolution as generalized protocols, where ethyl methane sulfonate (EMS) was used on wild-type strain W303-1A to

obtain a randomly mutagenized and genetically diverse initial population. The resulting population was used for 15 days Erlenmeyer flasks selections under higher glucose (100 g/L) and isobutanol (16 g/L) conditions throughout the cultivations. Individual mutant colonies are randomly selected from the final population and tested for their isobutanol tolerance using spot assay test. Meanwhile, relative viability rates were also determined. A strain designated as EMS39 with higher tolerance toward both glucose and isobutanol was identified. As shown in Fig.2a, both wild-type and the evolved strain EMS39 population could grow in control medium at all their dilutions (from  $10^0$  to  $10^{-4}$ ). However, wild type could barely grow at its  $10^0$  dilution in YPD with 16 g/L isobutanol (stress containing medium). Additionally, the evolved strain EMS39 could grow nearly at all of its dilutions ( $10^0$  to  $10^{-4}$ ) in YPD with 16 g/L isobutanol, which demonstrated its higher resistance properties against the stress factor. As shown in Fig.2b, the evolved strain EMS39 conferred a significantly improved cellular viability (over the course of 60 hours of culturing) above that of the control strain, even at concentrations as high as 20 g/L isobutanol. All these results indicated that the evolved strain EMS39 might be a predominant strain with higher tolerance toward isobutanol. So we used the evolved strain EMS39 as a platform to produce isobutanol.

### **Application of the evolved strain EMS39 as a platform for isobutanol production**

Metabolic engineering of the evolved strain EMS39 as a platform for isobutanol production were carried out. To over-express *ILV2*, *ILV5* and *ARO10*, 2 $\mu$  plasmids YEplac195-PGK1p-*ILV2*, YEplac112-PGK1p-*ILV5* and YEplac181-TDH3p-cox4-*ARO10* were transformed into the evolved strain EMS39, wild type strain W303-1A and strain HZAL-7 using LiAc/ssDNA/PEG methods, the resulting strains were designated as strain EMS39V2V5A10, strain W303-1AV2V5A10 and strain HZAL-7V2V5A10, respectively. And in order to increase the integrated copy number of *ILV3* (encoding enzyme dihydroxyacid dehydratase limiting the isobutanol pathway in *S.cerevisiae*),  $\delta$ -integration method was used to over-express *ILV3* in EMS39V2V5A10, W303-1AV2V5A10 and HZAL-7V2V5A10. And the resulting strains were donated as strain EMS39V2 $\delta$ V3V5A10, strain W303-1AV2 $\delta$ V3V5A10 and strain HZAL-7V2 $\delta$ V3V5A10, respectively. To determine whether the increased isobutanol tolerance of the evolved strain EMS39 could improve isobutanol yield, we examined performances of strain EMS39V2 $\delta$ V3V5A10, strain W303-1AV2 $\delta$ V3V5A10 and strain HZAL-7V2 $\delta$ V3V5A10 in micro-aerobic batch fermentation in YPD medium with 40 g L<sup>-1</sup> glucose and 130 g L<sup>-1</sup> glucose as carbon source in shaker flasks with OD<sub>600</sub>=0.5 and 3.0, as the initial inoculums size, respectively. And strain W303-1A $\delta$ His3 carrying plasmids YEplac181, YEplac195 and YEplac112 was used as the control strain.

As shown in Fig.3a and b, the control strain has the lowest growth rate and glucose consumption rate and glucose consumption was complete at 48h. Growth rate of strain EMS39V2 $\delta$ V3V5A10 was slightly higher than that of the control strain. And strain EMS39V2 $\delta$ V3V5A10 used up glucose at 32h. Meanwhile, growth rate of strain W303-1AV2 $\delta$ V3V5A10 and strain HZAL-7V2 $\delta$ V3V5A10 were higher than that of strain EMS39V2 $\delta$ V3V5A10. In addition, strain W303-1AV2 $\delta$ V3V5A10 and strain HZAL-7V2 $\delta$ V3V5A10 consumed glucose faster than strain EMS39V2 $\delta$ V3V5A10.

Isobutanol concentrations in the media at 24h, 28h, 32h, 36h and 48h after the start of cultivation were determined. As shown in Fig3.c, the control strain and strain W303-1A V2δV3V5A10 generated 0.064 g L<sup>-1</sup> and 0.299 g L<sup>-1</sup> isobutanol at 48h, respectively. While strain EMS39 V2δV3V5A10 generated 0.404 g L<sup>-1</sup> isobutanol at 32h. And strain HZAL-7 V2δV3V5A10 produced 0.384 g L<sup>-1</sup> isobutanol at 48h. Isobutanol titers of strain EMS39V2δV3V5A10 increased by 35.1% and 5.2% compared with that of strain W303-1AV2δV3V5A10 and strain HZAL-7V2δV3V5A10, respectively. These data suggested that the increased isobutanol tolerance of the evolved strain EMS39 was useful for improving isobutanol titers.

To further gain insights into fermentation characteristics of the evolved strain EMS39 and strain EMS39V2δV3V5A10 in higher glucose concentration, we carried out fermentations in YPD medium with 130 g L<sup>-1</sup> glucose in shaker flasks with OD<sub>600</sub>=3, as the initial inoculum size. As shown in Fig.4a and Fig.4b, the growth rate and glucose consumption of strain EMS39YEplac181YEplac195YEplac112 were markedly higher and faster than that of the other four strains. While strain EMS39 V2δV3V5A10 resulted in lower growth rate and glucose consumption rate than strain EMS39YEplac181YEplac195YEplac112. In addition, there were no obvious differences between the control strain and strain W303-1AV2δV3V5A10 in the growth rate and glucose consumption. Finally, the growth rate and glucose consumption rate of strain HZAL-7 V2δV3V5A10 were slightly lower than that of the control strain.

As shown in Fig.4c, the control strain and strain EMS39YEplac181YEplac195YEplac112 produced 0.807 g L<sup>-1</sup> isobutanol at 36h and 1.33 g L<sup>-1</sup> isobutanol at 32h, respectively. The increased isobutanol titers in strain EMS39YEplac181YEplac195YEplac112 further indicated that increased isobutanol tolerance was useful for improving isobutanol titers. Meanwhile, strain EMS39 V2δV3V5A10 generated 2.79 g L<sup>-1</sup> isobutanol at 24h. These results indicated that over-expression of *ILV2*, *ILV5*, *ILV3* and *ARO10* could increase isobutanol yield markedly. But after 24h, isobutanol titers of strain EMS39 V2δV3V5A10 decreased slightly. This perhaps due to the exhaustion of glucose. In addition, we found that strain W303-1AV2δV3V5A10 and strain HZAL-7V2δV3V5A10 gained 4.20 g L<sup>-1</sup> and 3.45 g L<sup>-1</sup> isobutanol at 48h, respectively. These results suggested that over-expression of *ILV2*, *ILV5*, *ILV3* and *ARO10* in strain W303-1A could markedly improve isobutanol titers. But over-expression of *BAT2* and deletion of *PDC6* is not useful for increasing isobutanol titers in strain W303-1A V2δV3V5A10.

Ethanol was one of the main byproducts in isobutanol fermentation in yeast. As shown in Fig.3d, the control strain and strain W303-1AV2δV3V5A10 generated 3.45g L<sup>-1</sup> ethanol at 48h and 3.51 g L<sup>-1</sup> ethanol at 36h, respectively. And the strain EMS39 V2δV3V5A10 produced 4.34 g L<sup>-1</sup> ethanol at 32h. While strain HZAL-7 V2δV3V5A10 produced 4.38 g L<sup>-1</sup> ethanol at 36h. The increased ethanol titers of strain EMS39 V2δV3V5A10 might be resulted from higher tolerance toward alcohols. While increased ethanol titers of strain HZAL-7 V2δV3V5A10 might be resulted from the deletion of *PDC6*. As illustrated in Fig.4d, the control strain produced 48.3 g L<sup>-1</sup> ethanol at 48h. Strain EMS39YEplac181 YEplac195YEplac112 and strain EMS39V2δV3V5A10 generated 49.7 g L<sup>-1</sup> and 46.2 g L<sup>-1</sup> ethanol at 28h, respectively. Meanwhile, strain W303-1A V2δV3V5A10 and strain HZAL-7 V2δV3V5A10 produced 47.0 g L<sup>-1</sup> ethanol at 36h and 45.2 g L<sup>-1</sup> ethanol at 48h, respectively. Ethanol titers of strain EMS39 YEplac181YEplac195YEplac112

and strain EMS39V2δV3V5A10 increased than that of strain W303-1A V2δV3V5A10 and strain HZAL-7V2δV3V5A10 in the first 32 h fermentation. These data suggested that the evolved strain EMS39 also might be a predominant strain for producing ethanol.

The highest isobutanol yields and productivities of these strains were also calculated (as shown in Table4). The highest isobutanol yields of strain W303-1AV2δV3V5A10, strain EMS39V2δV3V5A10, strain EMS39YEplac181YEplac195YEplac112 and strain HZAL-7V2δV3V5A10 were 31.7, 21.0, 10.2 and 26.2 mg per g glucose, respectively. And strain EMS39V2δV3V5A10 has the highest isobutanol productivity ( $0.116 \text{ g L}^{-1} \text{ h}^{-1}$ ). Meanwhile, ethanol yields of strain W303-1AV2δV3V5A10, strain EMS39V2δV3V5A10, strain EMS39YEplac181YEplac195YEplac112 and strain HZAL-7V2δV3V5A10 were 0.354, 0.347, 0.382 and 0.343 g per g glucose, respectively. All these results suggested that strain EMS39V2δV3V5A10 had advantages in isobutanol fermentation.

### **Preliminary investigation on the genetic basis of improved phenotype of evolved strain EMS39**

To identify the genetic basis of improved phenotype in the evolved strain EMS39, whole genome resequencing of the evolved strain EMS39 were carried out. More than 59 genes had mutations (including nucleotides insertions, nucleotides deletions and base changes) in their ORF or in upstream and downstream regulatory regions of genes. As shown in Table S1, twenty-six genes (including *GPR1*, *WSC2*, *APC1*, *CLB5*, *COS4*, *COS6*, *SOK2*, *FLO1*, *FLO5*, *FLO9*, *ASG1*, *AAD4*, *MTL1*, *MSS11*, *BUD27*, *PAF1*, *EPL1*, *TIR1*, *FIG2*, *RPL14A*, *RPS28B*, *SRP40*, *NGR1*, *NCL1*, *FAB1* and *ENT1*) had multi mutations in their ORF. These genes are involved in cell growth, Ras-cAMP pathway, MAPK signaling pathway, cell cycle, turnover of plasma membrane, pseudohyphal differentiation, flocculation, oxidative stress response, cell integrity signaling, basal transcription factors, translation initiation and elongation, autophagy, cell wall mannoprotein and adhesion, component of ribosomal subunits, ribosome assembly and function, RNA binding protein, RNA processing, phosphatidylinositol signaling system and endocytosis and so on. And six genes (including *COS4*, *SOD2*, *SPT6*, *RPL14A*, *PSR1*, *RAD2*) had multi-nucleotides insertions or deletions in their upstream regulatory regions. They are related to turnover of plasma membrane, protecting cells against oxygen toxicity and oxidative stress, transcription, chromatin maintenance, and RNA processing, component of ribosomal subunit, ribosome assembly and function, nucleotide excision repair and so on. Fourteen genes (including *WSC2*, *CDC20*, *CLB6*, *TUP1*, *SMC4*, *FLO1*, *TAF6*, *NIP1*, *RDH54*, *RPL14A*, *RPS28B*, *PSR1*, *NGR1*, *NCL1*) have mutations in the downstream regulatory regions. These results suggested that isobutanol tolerance required synergism of polygenic. But further investigations need to be carried out to explore mutations that can lead to improved isobutanol tolerance in *S.cerevisiae*.

### **EMS39V2δV3V5A10 resulted in transcription perturbations**

In order to investigate cellular transcription profile changes in strain EMS39V2δV3V5A10 in micro-aerobic fermentation with  $130 \text{ g L}^{-1}$ , samples were taken at 24 h for RNA sequencing, and strain W303-1AV2δV3V5A10 was used as the control strain. RNA-Seq-based transcriptomic analysis revealed cellular transcription profile changes resulting from EMS39. The volcano plots of differentially expressed genes

(DEGs) for W303-1AV2δV3V5A10 vs EMS39V2δV3V5A10 were shown in Fig.5. Compared with strain W303-1AV2δV3V5A10, 401 differentially expressed genes (DEGs) were identified in strain EMS39V2δV3V5A10 (fold change > 2, P value < 0.05), including 160 up-regulated and 241 down-regulated.

Firstly, we reconstructed the central carbon metabolic network based on the RPKM values of genes involved in this network (Fig. 6 and Table S2). Glucose is phosphorylated by hexose–glucose kinase after uptake, and then enters the glycolytic pathway. The high-affinity glucose transporter genes *HXT6* and *HXT7* in strain EMS39V2δV3V5A10 were up-regulated by 8.1-fold and 11.5-fold compared with that in W303-1A V2δV3V5A10, respectively. The up-regulation of *HXT6* and *HXT7* in strain EMS39V2δV3V5A10 might promote its glucose assimilation ability. Transcriptional levels of gene *TPS2* and *TSL1* in strain EMS39V2δV3V5A10 increased by 8.1-fold 5.7-fold, respectively. The increased transcriptional level of *TSL1* and *TPS1* might resulted in accumulation of trehalose, which could increase the stability of the cells and stimulates the secretion of heat shock proteins [34]. *SOL4* and *SOL1* were up-regulated in strain EMS39V2δV3V5A10. These two genes encode 6-phosphogluconolactonase in the pentose phosphate pathway. The enhanced pentose phosphate pathway activity perhaps might provide a large amount of nicotinamide adenine dinucleotide phosphate (NADPH) for isobutanol biosynthesis. *FBP1*, encoding fructose 1,6-bisphosphatase, was found up-regulated by 16.4-fold. *ERR2*, encoding a phosphopyruvate hydratase, was down-regulated by 17.2-fold. *PCK1*, encoding phosphoenolpyruvate carboxykinase, was up-regulated by 17.9-fold. The perturbations of *FBP1*, *ERR2* and *PCK1* perhaps could promote regeneration of 2-phosphoglycerate. It was reported that *ADH1* knockdowns conferred increased tolerance toward both isobutanol and 1-butanol[35]. While our result indicated that alcohol dehydrogenase genes *ADH1* and *ADH5* were up-regulated by 2.8-fold and 3.0-fold in strain EMS39V2δV3V5A10, respectively. But alcohol dehydrogenase gene *ADH4* was down-regulated by more than 42-fold. *ADH1* is required for the reduction of acetaldehyde to ethanol, while *ADH4* is involved in the degradation of ethanol and thereby contribute to ethanol detoxification to ensure cell survival. We found that FPKM values of *ADH1* increased by 78.1-fold compared with that of *ADH4*. Hence, the enhanced transcription of *ADH1* in strain EMS39V2δV3V5A10 might confer it higher ethanol biogenesis. *MPC3*, encoding the highly conserved subunit of mitochondrial pyruvate carrier, was up-regulated by 33.2-fold in strain EMS39V2δV3V5A10. The up-regulation of *MPC3* might promote pyruvate uptake into mitochondrial matrix. *CIT1* was found up-regulated by 6.7-fold in strain EMS39V2δV3V5A10. Cit1p catalyzes the first reaction of the TCA cycle that is condensation of acetyl-CoA and oxaloacetate to form citrate. And Cit1p functions as a rate-limiting enzyme of the TCA cycle [36]. *IDP2*, encoding cytosolic NADP-specific isocitrate dehydrogenase Idp2 in the TCA cycle, was also up-regulated in strain EMS39V2δV3V5A10. The up-regulation of genes *CIT1* and *IDP2* might enhance the activity of TCA cycle, Under anaerobic conditions, the TCA cycle can work as a reducing cycle, reducing the excess NADH[37]. Additionally, *MLS1*, encoding malate synthase in the glyoxylate cycle, was also up-regulated by 9.4-fold in strain EMS39V2δV3V5A10. The up-regulation of *MLS1* perhaps could improve utilization of acetyl CoA. *GLO1* and *CYB2*, encoding glyoxalase I and L-lactate dehydrogenase, were up-regulated by 5.9-fold and 6.9-fold in strain EMS39V2δV3V5A10, respectively. The enhanced transcription of *GLO1* and *CYB2* perhaps could improve pyruvate biogenesis. Transcriptional levels of *ILV2*, *ILV3* and *ADH7* were up-

regulated by 3.3-fold, 2.1-fold and 8.8-fold in strain EMS39V2δV3V5A10, respectively. The up-regulations of *ILV2*, *ILV3* and *ADH7* conferred strain EMS39V2δV3V5A10 higher isobutanol titers. But transcriptional level of *ARO10* decreased by more than 90-fold. This indicated that there still existed transcription unbalance of genes involved in isobutanol biosynthesis.

Secondly, the RPKM values of genes related to transporters of the plasma membrane and mitochondrial membrane were analyzed. *PMA2*, encoding plasma membrane H<sup>+</sup>-ATPase, involved in pumping protons out of the cell and it was the regulator of cytoplasmic pH and plasma membrane potential. The down-regulation of *PMA2* might decrease the pumping protons out of the cell and increase pH gradient across the membrane in strain EMS39V2δV3V5A10. *JEN1*, encoding monocarboxylate/proton symporter Jen1p of the plasma membrane, was up-regulated by 6.4-fold in strain EMS39V2δV3V5A10. Jen1p mediates high-affinity uptake of carbon sources lactate, pyruvate, and acetate, and also of the micronutrient selenite. And transport activity of Jen1p is dependent on the pH gradient across the membrane. The increased pH gradient across the membrane, which was due to the down-regulation of *PMA2* in strain EMS39V2δV3V5A10, perhaps resulted in up-regulation of *JEN1*. *STL1*, encoding glycerol proton symporter of the plasma membrane, was up-regulated by 5.3-fold in strain EMS39V2δV3V5A10. This gene was subject to glucose-induced inactivation and was strongly but transiently induced when cells are subjected to osmotic shock. The up-regulation of *STL1* in strain EMS39V2δV3V5A10 might confer it higher osmotic tolerance. While *PHO84*, *PHO89*, *FRE1*, *FRE7*, *FET4*, *MRS3*, *ZRG17*, *IZH2*, *ZRT1* and *ZRT2* were down-regulated in strain EMS39V2δV3V5A10. The down-regulation of *PHO84* and *PHO89* might decrease the phosphate ion transmembrane transport and polyphosphate metabolism. The down-regulation of *FRE1*, *FRE7*, *FET4*, *MRS3* might maintain iron ion homeostasis in cytoplasmic and mitochondria. And the down-regulation of *ZRG17*, *IZH2*, *ZRT1* and *ZRT2* might maintain zinc ion homeostasis in endoplasmic reticulum and cytoplasmic. In addition, the down-regulated genes related to iron ion homeostasis and zinc ion homeostasis perhaps indicated the decreased synthesis of iron ion-containing amino acids and zinc ion-containing amino acids in strain EMS39V2δV3V5A10. *SFC1*, encoding mitochondrial succinate-fumarate transporter Sfc1, was up-regulated by 17.5-fold in strain EMS39V2δV3V5A10. Sfc1 is involved in transporting succinate into and fumarate out of the mitochondrion. We also found that *UPS2*, *UPS3* and *ODC1* were up-regulated by 4.9-fold, 3.4-fold and 3.8-fold in strain EMS39V2δV3V5A10, respectively. *UPS2*, encoding mitochondrial intermembrane space protein, was involved in phospholipid metabolism. *ODC1*, encoding 2-oxodicarboxylate transporter on mitochondrial inner membrane, exports 2-oxoadipate and 2-oxoglutarate from the mitochondrial matrix to the cytosol for lysine and glutamate biosynthesis and lysine catabolism. The up-regulation of *UPS2* perhaps could improve activity of phospholipid metabolism. And the up-regulation of *ODC1* perhaps could improve lysine and glutamate biosynthesis and lysine catabolism. *TPC1*, encoding mitochondrial membrane transporter, was down-regulated by 3.2-fold in strain EMS39V2δV3V5A10. The down-regulation of *TPC1* might decreased the uptake of the essential cofactor thiamine pyrophosphate (ThPP) into mitochondria.

Thirdly, other groups up-regulated DEGs in strain EMS39V2δV3V5A10 are related to thiamine metabolism (i.e. *THI12*, *THI5*, *THI11*, *THI13*), heat shock proteins (such as *HSP26*, *HSP30* and *HSP10*), response to oxidative stress (such as *STF2*), oxidative phosphorylation (such as *QCR9*, *QCR8*, *COX7*, *QCR10*, *COX12*, *COX6*, *RIP1* and *ATP20*), cytochrome c (i.e. *CYC1*, *CYC7*, *PET100*), some transcription factors (such as *MET28*, *HAP4* and *MET32*), adenylylsulfate kinase (i.e. *MET14*) and SNARE- and chaperone-binding protein (i.e. *BTN2*) (Table S2). Thiamine and its phosphate compounds are required by all organisms to participate in various cellular metabolism. It was reported that the production of thiamine played an important role in the stress of oxidative stress [34,38]. The up-regulation of genes involved in thiamine metabolism and response to oxidative stress in strain EMS39 V2δV3V5A10 might improve its oxidative stress tolerance. The up-regulation of genes involved in heat shock proteins, SNARE- and chaperone-binding protein might improve isobutanol tolerance and ethanol tolerance of strain EMS39 V2δV3V5A10. The up-regulation of genes involved in oxidative phosphorylation and cytochrome c might supply cells with more energy. Another group of genes *MET5*, *MET13*, *MET14*, *MHT1* and *YCT1* were up-regulated in strain EMS39 V2δV3V5A10. These genes are involved in sulfur assimilation and are associated with methionine/cysteine metabolism. Now the roles of amino acids in the alcohol tolerance regulatory network is not yet clear. It was speculated that the synthesis of sulfur-containing amino acids was increased in order to increase the sulfur reserve in advance to ensure the subsequent synthesis of glutathione (GSH) [39]. Other groups down-regulated DEGs in strain EMS39V2δV3V5A10 are involved in biotin metabolism (i.e. *BIO3*, *BIO4*, *BIO5*), the vacuolar transporter chaperone complex (i.e. *VTC1*), ribosome biogenesis (such as *NHP2*, *CBF5*, *NOP58*, *NOP1*, *GAR1*, *NOG2*, *NOP4*, *NOG1*, *UTP14*, *EMG1*, *UTP5*, *DIP2*, *UTP21*, *PWP2*, *UTP10*, *UTP13*, *KRE33*, *UTP4*, *RIX7*, *UTP8*), purine metabolism (i.e. *ADE17*, *AAH1*, *ADE4*), Vitamin B<sub>6</sub> metabolism (i.e. *BUD17* and *SNO1*), putative aryl alcohol dehydrogenase (i.e. *AAD16*) and drug metabolism (i.e. *IMD4*, *GUA1*, *IMD2*, *IMD3*). Down-regulation of genes involved in ribosome biogenesis, purine metabolism, vitamin metabolism, putative aryl alcohol dehydrogenase and drug metabolism in strains with higher tolerance toward alcohols were found by previous report [35,40,41]. Intriguingly, down regulation of biotin metabolism and regulatory subunit of the vacuolar transporter chaperone (VTC) complex coding gene *VTC1* were only found in our study. Biotin function as cofactors of carboxylase involved in carbon dioxide transfer during respiration. The down-regulated biotin metabolism in strain EMS39V2δV3V5A10 indicated the decreased activity of carboxylase. The down-regulated gene *VTC1* perhaps indicated decreased membrane trafficking and DNA replication stress in strain EMS39V2δV3V5A10.

To further explore the transcriptome perturbations caused by EMS39, we conducted differential expression analysis based on the RNA-Seq data. Gene Ontology (GO) (Additional file 4) and KEGG (Additional file 5) enrichment analysis were conducted to identify the functions of DEGs. The results of GO enrichment show that biological processes related to gluconeogenesis (GO:0006094), cell division (GO:0051301), DNA integration (GO:0015074), rRNA processing (GO:0006364), ribosome biogenesis (GO:0042254), pseudouridine synthesis (GO:0001522) and zinc ion transmembrane transport (GO:0071577) were enriched in the down-regulated DEGs (Additional file 4). And biological processes related to trehalose biosynthetic process (GO:0005992) were enriched in the up-regulated DEGs. In

addition, cellular components related to ribonucleoprotein complex (GO:0030529), phosphopyruvate hydratase complex (GO:0000015), vacuolar transporter chaperone complex (GO:0033254) and intracellular part (GO:0044424) were enriched in the down-regulated DEGs. Furthermore, molecular functions related to RNA binding (GO:0003723), pseudouridine synthase activity (GO:0009982), aspartic-type endopeptidase activity (GO:0004190), IMP dehydrogenase activity (GO:0003938), hydrogen ion transmembrane transporter activity (GO:0015078), phosphopyruvate hydratase activity (GO:0004634), zinc ion transmembrane transporter activity (GO:0005385) and L-serine ammonia-lyase activity (GO:0003941) were enriched in the down-regulated DEGs. According to the KEGG enrichment result (Additional file 5), apoptosis (ko04215), sulfur metabolism (ko00920), oxidative phosphorylation (ko00190, ko04260) and thiamine metabolism (ko00730) were enriched in the up-regulated DEGs in strain EMS39 V2δV3V5A10. Regarding the enrichment analysis of the down-regulated DEGs, the biological processes related to ribosome biogenesis, biotin metabolism, drug metabolism and purine metabolism were enriched in strain EMS39 V2δV3V5A10. In previous studies, GO analysis revealed the following GO terms to be overrepresented in the DEGs in alcohol-tolerant strains: gluconeogenesis, cell division, DNA integration, rRNA processing, ribosome biogenesis, sulfur metabolism, oxidative phosphorylation, vitamin metabolism, electron transport and trehalose biosynthetic process[35, 37, 40-42]. Intriguingly, down-regulation of genes involved in zinc ion transmembrane transport and vacuolar transporter chaperone complex were only found in this study.

## Discussion

Isobutanol would be an ideal substitute for fossil fuels, because they have high-energy density and low hygroscopicity and can drop in directly in the current infrastructure and engines, preferred especially in aviation and diesel fuels. *S. cerevisiae* can produce low amount of isobutanol via valine synthesis pathway. However, isobutanol is toxic to yeast cells, lowering the efficiency and cost-effectiveness of these processes. So strain's isobutanol tolerance is one of important factors that restrict improvements of isobutanol fermentation performance in *S. cerevisiae*. The evolution of such complex traits requires simultaneous modification in many genes' expression levels. Adaptive laboratory evolution could be used to screen desired phenotype that is generally dependent on multiple factors, which could not be identified unless genome-wide or large-scale approaches are used [25–26]. In this paper, we employed adaptive laboratory evolution strategies to obtain enhanced isobutanol and glucose stress-resistant *S. cerevisiae* mutants. And a strain designated as EMS39 was identified. Our results showed that the evolved strain EMS39 has higher isobutanol tolerance and improved cellular viability (Fig. 2). These results suggested that adaptive laboratory evolution could be used to screen mutants with higher tolerance toward both isobutanol and glucose.

To investigate whether the enhanced tolerance toward isobutanol is useful to increase isobutanol yield, fermentation characteristics of strain EMS39V2δV3V5A10 in micro-aerobic batch fermentation with 40 g L<sup>-1</sup> initial glucose and 130 g L<sup>-1</sup> initial glucose were investigated. Our data suggested that strain EMS39YEplac181YEplac195YEplac112 and strain EMS39V2δV3V5A10 had higher growth rates

compared to the control strain and strain W303-1AV2 $\delta$ V3V5A10 in fermentation with 130 g L<sup>-1</sup> initial glucose. In addition, glucose consumption rates of strain EMS39YEplac181YEplac195YEplac112 and strain EMS39V2 $\delta$ V3V5A10 were higher than the other three strains. This indicated that the evolved strain EMS39 had higher adaptability in fermentation with higher initial glucose. Meanwhile, strain EMS39V2 $\delta$ V3V5A10 produced higher isobutanol titers in micro-aerobic batch fermentation and in the first 24 h of fermentation with 130 g L<sup>-1</sup> initial glucose. And strain EMS39V2 $\delta$ V3V5A10 gained the highest isobutanol productivity. Our data also shown that strain EMS39YEplac181YEplac195YEplac112 and strain EMS39V2 $\delta$ V3V5A10 produced higher ethanol titers. All these indicated that the evolved strain EMS39 might be a high-efficiency predominant strain for isobutanol fermentation and ethanol fermentation. In addition, our results suggested that improving yeast strain's isobutanol tolerance was useful to increase its ability to produce isobutanol.

We have reported that deletion of PDC6 could improve isobutanol yield [9]. And we also reported that strain pILV2pARO10(W303-1AV2A10) produced 2.98 mg isobutanol per g glucose [13]. Here, we demonstrated that strain W303-1AV2 $\delta$ V3V5A10 produced 31.7 mg isobutanol per g glucose. Isobutanol yield of strain W303-1AV2 $\delta$ V3V5A10 increased 10.6-fold compared to that of strain pILV2pARO10(W303-1AV2A10). This indicated that over-expressing of ILV3 (encoding enzyme dihydroxyacid dehydratase limiting the isobutanol pathway) by using  $\delta$ -integration method could markedly increase isobutanol yield.

To investigate the genetic basis of improved phenotype in the evolved strain EMS39, whole genome resequence of the evolved strain EMS39 were carried out. Preliminary investigation of whole genome resequencing of evolved EMS39 suggested that twenty-six genes (including GPR1, WSC2, APC1, CLB5, COS4, COS6, SOK2, FLO1, FLO5, FLO9, ASG1, AAD4, MTL1, MSS11, BUD27, PAF1, EPL1, TIR1, Fig. 2, RPL14A, RPS28B, SRP40, NGR1, NCL1, FAB1 and ENT1) had multi base mutations in their ORF, six genes (including COS4, SOD2, SPT6, RPL14A, PSR1, RAD2) had multi base mutations in their upstream regulatory regions, and fourteen genes (including WSC2, CDC20, CLB6, TUP1, SMC4, FLO1, TAF6, NIP1, RDH54, RPL14A, RPS28B, PSR1, NGR1, NCL1) have multi mutations in the downstream regulatory regions. These results indicated that tolerance toward isobutanol does not a monogenic trait, it required multiple genes working together corporately. Furthermore, mutations in GPR1 perhaps conferred to the higher growth rate and higher glucose consumption rate of the evolved strain EMS39. Because GPR1 acts as G protein-coupled receptor that senses glucose and controls filamentous growth, and it is an important protein in Ras-cAMP pathway that regulate multi-aspects of cell growth. In addition, mutations in AAD4 and SOD2 (encoding putative aryl-alcohol dehydrogenase and mitochondrial manganese superoxide dismutase, respectively) perhaps conferred to higher isobutanol tolerance in the evolved strain EMS39. Furthermore, mutations in ASG1, TAF6 and MSS11 perhaps acted as transcriptional regulators or transcription factors to regulate multi-aspects of cell growth in the evolved strain EMS39. All these genes might work together corporately to confer the evolved strain EMS39 improved isobutanol tolerance.

To investigate the perturbations of transcriptome resulting from EMS39, we conducted differential expression analysis based on the RNA-Seq data. Compared with control strain (W303-1AV2 $\delta$ V3V5A10), strain EMS39 V2 $\delta$ V3V5A10 had 401 differentially expressed genes (DEGs) (fold change > 2, P value <

0.05), including 160 up-regulated and 241 down-regulated. The up-regulated DEGs in strain EMS39 V2δV3V5A10 are related to the central carbon metabolic network, trehalose biosynthetic process, thiamine metabolism, heat shock proteins, response to oxidative stress, oxidative phosphorylation, apoptosis and sulfur metabolism. While the down-regulated DEGs in strain EMS39 V2δV3V5A10 are related to ribosome biogenesis, biotin metabolism, purine metabolism, Vitamin B<sub>6</sub> metabolism, drug metabolism, putative aryl alcohol dehydrogenase, and regulatory subunit of the vacuolar transporter chaperone complex. Up-regulation of genes in strain EMS39 V2δV3V5A10 related to the central carbon metabolic network perhaps confer it higher growth rate and glucose consumption rate. Up-regulation of genes in strain EMS39 V2δV3V5A10 involved in trehalose biosynthetic process and oxidative stress response in strain EMS39V2δV3V5A10 perhaps confer it higher isobutanol tolerance. It was reported that knockdown of Hsp70p heat shock proteins improves isobutanol tolerance [35]. In the present study, we found that three heat shock proteins were up-regulated in strain EMS39V2δV3V5A10. One is Hsp10p that is involved in maintaining the stability of proteins in the mitochondria, ensuring their correct folding. The other one is Hsp26p that acts on the cytoplasm to prevent irreversible aggregation of proteins, which cannot properly folded. The third is Hsp30p that focuses on proteins on the plasma membrane and regulates the plasma membrane H<sup>+</sup>-ATPase to prevent rapid depletion of energy in the cell. The increased transcriptional levels of these heat shock proteins might be important adaptation mechanisms in *S. cerevisiae*, which could be induced by a series of intracellular adjustments. In addition, down regulation of biotin metabolism and regulatory subunit of the vacuolar transporter chaperone (VTC) complex coding gene VTC1 were only found in our study. Furthermore, Gene Ontology (GO) and KEGG enrichment analysis suggested that down-regulation of genes involved in zinc ion transmembrane transport and vacuolar transporter chaperone complex were only found in this study. The down-regulation of zinc ion transmembrane transporter activity perhaps indicated the decreased synthesis of zinc ion-containing amino acids in strain EMS39V2δV3V5A10. The down-regulated gene VTC1 perhaps indicated decreased membrane trafficking and DNA replication stress in strain EMS39V2δV3V5A10. Finally, all these genes' transcriptional perturbations might work together corporately to confer strain EMS39V2δV3V5A10 improved isobutanol titers. It was reported that mutations in the eIF2 and eIF2B complexes greatly improve tolerance to these medium-chain alcohols[41]. While other studies had examined effects of overexpression of a single gene or individual gene knockout on the phenotype of improved alcohol tolerance[40, 46, 47]. Their results suggested that each gene was insufficient to confer alcohol tolerance individually. Our results suggested that multi genes' transcriptional perturbations might work together corporately to confer strain EMS39 increased isobutanol tolerance and strain EMS39V2δV3V5A10 improved isobutanol titers.

## Conclusions

In this study, we successfully obtained the evolved strain EMS39 that had enhanced tolerance toward higher isobutanol and glucose concentration by EMS (ethyl methanesulfonate) mutagenesis followed by adaptive laboratory evolution. In addition, metabolic engineering methods were used to improve isobutanol yield. Further, we gained higher isobutanol titers (4.20 g L<sup>-1</sup>) and higher isobutanol

productivity (0.116 g/L/h). Finally, preliminary investigation of whole genome resequencing of the evolved strain EMS39 revealed the molecular mechanisms of mutations affecting isobutanol tolerance in *S.cerevisiae*. And analysis of transcriptional perturbations in strain W303-1AV2 $\delta$ V3V5A10 and strain EMS39 V2 $\delta$ V3V5A10 revealed transcriptional perturbations of relative genes conferred yeast *S.cerevisiae* increased isobutanol tolerance and improved strain's ability to produce isobutanol.

## Methods

### Yeast strains and growth conditions

W303-1A was used as the parental strain and all strains used in this study and their genotypes were listed in Table 1. Plasmids and their descriptions were showed in Table 2. *Escherichia coli DH5a* was used as the cloning host and recombinant strains were cultured at 37°C in Luria-bertani medium (LB) (1% tryptone, 0.5% yeast extract and 1% NaCl) with 100  $\mu$ g/mL ampicillin. Yeast extract peptone dextrose (YPD) medium (2% peptone, 1% yeast extract, 2% glucose) was used to routinely maintain and propagate yeast strains. Synthetic complete (SC) media (0.67% bacto yeast nitrogen base without amino acids supplemented with appropriate amino acid and 2% D-glucose) were used for selection of transformants.

### DNA manipulation, plasmids and strains construction

Standard molecular genetic techniques were used for nucleic acid manipulations [43]. Primers used in this study were listed in Table 3. DNA templates used for PCR amplification of yeast genomic sequences were isolated from strain W303-1A. Transformation of yeast cells was carried out with using LiAc/ssDNA/PEG method.

Methods for over-expressing *BAT2* and *ILV2* and knock-out of *PDC6* gene were illustrated in our previous report [9] (Zhang A.L. *et al.* 2016). And methods for over-expression of *ARO10* were illustrated in our previous report [13]. In order to over-express *ILV5*, plasmid YEplac112-PGK1p-*ILV5* was constructed. *ILV5* was amplified by PCR using primers *ILV5ORF-U* (containing *Sal*I enzyme site) and *ILV5ORF-D* (containing *Pst*I enzyme site). PGK1p was digested by endonuclease *Bam*H I and *Sal*I and inserted in front of *ILV5* in plasmid YEplac112. Plasmid YEplac112-PGK1p-*ILV5* was introduced into mutants using the LiAc/ssDNA/PEG method and transformants were selected on SC minus tryptophan medium.

$\delta$ -integration method was used to over-express *ILV3*. Firstly, plasmid pUC18- $\delta$ 5'-PGK1p-*ILV3*-His3- $\delta$ 3' was constructed.  $\delta$ 5' was amplified by PCR using primers *Del1-U* and *Del1-D*. *ILV3* was amplified by PCR using primers *ILV3ORF-U* (containing *Sal*I enzyme site) and *ILV3ORF-D* (containing *Pst*I enzyme site). PGK1p was digested by endonuclease *Bam*H I and *Sal*I and inserted in front of *ILV3*. *HIS3* was amplified by PCR using primers *HIS3-U* (containing *Xba*I enzyme site) and *HIS3-D* (containing *Sal*I enzyme site).  $\delta$ 3' was amplified by PCR using primers *Del2-U* and *Del2-D*. Then, *Hind*III and *Eco*R I were used to digested plasmid pUC18- $\delta$ 5' -PGK1p-*ILV3*-His3- $\delta$ 3' and DNA fragment  $\delta$ 5'-PGK1p-*ILV3*-His3- $\delta$ 3' was purified with DNA Purification Kit. Finally, DNA fragment  $\delta$ 5'-PGK1p-*ILV3*-His3- $\delta$ 3' was transformed into EMS39 using LiAc/ssDNA/PEG method and transformants were selected on SC minus histidine medium.

## Mutagenesis by EMS treatment

1 mL fresh incubated cells ( $2 \text{ OD}_{600\text{nm}}$ ) were collected by centrifugation, washed twice with 0.1 M potassium phosphate buffer (pH 7.0) and resuspended in 2 mL potassium phosphate buffer. Then four percent (v/v) EMS that gave rise to 85% lethality was added and mixed with the cell suspension by vortexing vigorously. The mixture was then incubated for 45 min at 30 °C with gentle agitation. And the mutagenesis was stopped by adding an equal volume of freshly made 5% (w/v) sodium thiosulfate. Finally, the mutagenized cells were collected by centrifugation, washed twice with 5% (w/v) sodium thiosulfate, and resuspended in sterile ddH<sub>2</sub>O for selection or stored in -80 °C with 15% (v/v) glycerol.

## Adaptive laboratory evolution

Adaptive laboratory evolution was performed by a serial batch transfer procedures and by batch fermentation in a 100 ml Erlenmeyer flasks in the presence of 16 g/L isobutanol as a selective pressure. Firstly, a single micro-aerobic culture of mutagenized W303-1A was initiated in 10 ml YPD supplemented with 16 g/L isobutanol in 50 ml Erlenmeyer flasks and grown at 30 °C and 150 rpm for 24 hours. Then this culture was passaged daily in fresh YPD with 16 g/L isobutanol. After 15 daily passages, a diluted aliquot of the final culture was plated for single colonies on YPD plates with 16 g/L isobutanol. Finally, isobutanol tolerance of yeast strains derived from a number of single colonies were tested (see “Spot Assay Test” below) and compared with the parental strain. The best performing strain was designated as EMS39.

## Spot Assay Test

Firstly, 1 mL fresh incubated cells ( $1 \text{ OD}_{600\text{nm}}$ ) were collected by centrifugation, washed once with sterile ddH<sub>2</sub>O and resuspended in 1 mL sterile ddH<sub>2</sub>O. Then, for each culture, prepare  $10^0$ ,  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  fold dilutions and spot 5 $\mu$ L of each dilution onto YPD plates (as the control plates) and YPD plates with 16 g/L isobutanol (as the stress containing plates), respectively. Finally, incubate the plates for 2-3 days at 30 °C and observe the growth of the cultures (Fig. 1).

## Viability curve assays

Cells were incubated in 10 ml YPD medium with 20 g/L glucose in 100 ml Erlenmeyer flasks for 16 hours at 30°C. Then, approximately 5 ml were centrifuged at 3000 rpm for 5 minutes. And cell pellets were resuspended in 30 ml YPD with 20 g/L of glucose and 16 g/L isobutanol to a final cell concentration of  $\text{OD}_{600}=1$ , and incubated at 30°C with 200 rpm orbital shaking. 100  $\mu$ L samples were collected (following vortexing to ensure homogeneity) every four hours (including the zero time point) and diluted and plated onto YPD plates. Finally, these YPD plates were then incubated for 2-3 days to allow for colony formation and colony forming unit counts. Both control strains and the mutant were incubated in biological replicate.

## Isobutanol fermentation and metabolite analysis

All micro-aerobic batch fermentation cultivations were carried out in 250 mL cap-covered Erlenmeyer flasks with a working volume of 100 mL medium. Inoculums were cultured in SC medium (YNB without amino acids (Difco) supplemented with 20 g L<sup>-1</sup> glucose and amino acids according to strains demands) until OD<sub>600nm</sub> ≈ 5.0 in 100 mL Erlenmeyer flasks at 30°C. Then, cells were collected and used to inoculate 100 mL YPD medium with 40 g L<sup>-1</sup> or 130 g L<sup>-1</sup> glucose in Erlenmeyer flasks with OD<sub>600</sub> = 0.5 (or 3.0), as the initial inoculums size. During fermentations, the flasks were kept at 30°C with 100 rpm agitating to create micro-aerobic conditions. Samples were collected during fermentation.

Methods for measurements of cell growth, glucose, isobutanol and ethanol were illustrated in our previous report (Zhang A.L. *et al.* 2016).

### **Whole genome resequencing**

The evolved strain EMS39 was used for whole genome resequencing to identify important mutations occurred in the evolution. Strains were recovered with YPD medium from frozen stock for genomic DNA extraction with wild type strain W303-1A as a control. Genomic DNA were prepared via a standard phenol-chloroform method. Then genome DNA prepared was sent to Biomarker Technologies (Beijing, China) for library construction for next-generation sequencing according to the standard procedure of genome resequencing pipeline. Detailed procedures were carried out as described below. Firstly, qualified genomic DNA was then sheared on a Bioruptor Pico System (Diagenode, Belgium) to average length of 350bp. Then overhangs generated from the prepared fragmentation were repaired and an “A” base was added to the 3’ end of the blunt phosphorylated DNA fragments; adapters were then ligated to the ends of the DNA fragments by using End Rep Enzyme Mix (ExCellBio, Shanghai, China). The desired fragments were purified by using DNA clean beads (Vazyme Biotech Co.,Ltd, Nanjing, China) and selectively enriched by PCR amplification. Indexed tags were introduced into the adapters at the PCR stage. The libraries were prepared with TrueLib DNA Library Rapid Prep Kit (Illumina, San Diego, CA, USA). And the qualified libraries were used for next-generation sequencing via the Illumina HiSeq X-ten platform (San Diego, CA, USA). Clean reads were purified from raw data by removing low-quality reads and then mapped to the *S.cerevisiae* S288C genome (R64-1-1.20110203.tgz) obtained from Saccharomyces Genome Database genome using bwa software (Version: 0.7.10-r789) [44]. The mapped reads were then sorted and duplicated reads were marked using Picard (Version: 1.94(1484)). Single Nucleotide variation (SNV)/InDel calling was done using the HaplotypeCaller algorithm of GATK, and the SnpEff output was used to decipher the potential effects of the mutations [45]. The distribution of variation results of different types is shown by using Circos diagram. Bioinformatics analysis was done using NR database, SwissProt database, GO database, COG database and KEGG database.

### **Transcriptome sequencing**

Strains W303-1AV2δV3V5A10 and EMS39 V2δV3V5A10 were used for transcriptome sequencing to identify genes’ transcriptional levels and wild type strain W303-1A was used as a control. Samples were sent to Genewiz (Suzhou, China) for next-generation sequencing via the Illumina HiSeq X-ten platform

(San Diego, CA, USA) according to the standard procedure of genome resequencing pipeline. Firstly, samples were recovered from frozen stock for total RNA extraction via TRIzol method. Then the poly(A) mRNA isolation was performed using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB). The mRNA fragmentation and priming was performed using NEBNext First Strand Synthesis Reaction Buffer and NEBNext Random Primers. First strand cDNA was synthesized using ProtoScript II Reverse Transcriptase and the second-strand cDNA was synthesized using Second Strand Synthesis Enzyme Mix. The purified double-stranded cDNA by AxyPrep Mag PCR Clean-up (Axygen) was then treated with End Prep Enzyme Mix to repair both ends and add a dA-tailing in one reaction, followed by a T-A ligation to add adaptors to both ends. Size selection of Adaptor-ligated DNA was then performed using AxyPrep Mag PCR Clean-up (Axygen), and fragments of ~360 bp (with the approximate insert size of 300 bp) were recovered. Each sample was then amplified by PCR for 11 cycles using P5 and P7 primers, with both primers carrying sequences which can anneal with flow cell to perform bridge PCR and P7 primer carrying a six-base index allowing for multiplexing. The PCR products were cleaned up using AxyPrep Mag PCR Clean-up (Axygen), validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Sequencing was carried out using a 2x150bp paired-end (PE) configuration; image analysis and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument.

The software fastqc (v0.10.1) was used to evaluate the quality of raw reads, and the software Cutadapt [version 1.9.1] was used to filter low quality reads and remove pollution and joint sequence. Then clean reads were mapped to the *S.cerevisiae* S288C genome (R64-1-1.89) obtained from ENSEMBL Database by using software Hisat2(v2.0.1). Variable shear analysis was done by using the software ASprofile [V1.0.4]. Samtools (v0.1.19) software was used to perform mpileup processing through the comparison results of each sample and reference genome, so as to obtain the possible SNV results of each sample, and then annotate them with annovar (v2013.02.11) software. The gene expression level and gene difference analysis were calculated by using the software cuffdiff (v2.2.1), which used FPKM [Fragments Per Kilo bases per Million reads] method to calculate gene expression. Bioinformatics analysis was done using GO database and KEGG database. Differential exon usage was done by using DEXSeq [V1.18.4] software.

## Declarations

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## Authors' contributions

JL and AZ designed the experiments; YS and WZ performed the experiments and analyzed the results; AZ and YS wrote the manuscript. AZ supervised the work. All authors read and approved the final manuscript.

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## Availability of data and materials

The materials and data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## Abbreviations

EMS: ethyl methanesulfonate; HMP: hydroxymethylpyrimidine.

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## Tables

Table 1 Strains used in this study

Strains	Genotypes	Sources
03-1A	<i>MATa leu2-3, 112 ura3-1 trp1-92 his-11, 15 ade2-1 can1-100</i>	Thomas and Rothstein, 1989
03-1A $\delta$ His3	<i>MATa leu2-3, 112 ura3-1 trp1-92 his-11, 15 ade2-1 can1-100, 65'-His3-63'</i>	This work
S39	unknown	This work
AL-7	W303-1A <i>PGK1p-BAT2 pdc6<math>\Delta</math>::R</i>	(Zhang <i>et al.</i> 2016)
03- $\delta$ V3V5A10	W303-1A 65'- <i>PGK1p-ILV3-His3-63'</i> YEplac181- <i>TDH3p-Cox4-ARO10</i> YEplac195- <i>PGK1p-ILV2</i> YEplac112- <i>PGK1p-ILV5</i>	This work
S39	EMS39 65'- <i>PGK1p-ILV3-His3-63'</i> YEplac181- <i>TDH3p-Cox4-ARO10</i> YEplac195- <i>PGK1p-ILV2</i> YEplac112- <i>PGK1p-ILV5</i>	This work
$\delta$ V3V5A10	ARO10 YEplac195- <i>PGK1p-ILV2</i> YEplac112- <i>PGK1p-ILV5</i>	
AL-7	HZAL-7 65'- <i>PGK1p-ILV3-His3-63'</i> YEplac181- <i>TDH3p-Cox4-ARO10</i> YEplac195- <i>PGK1p-ILV2</i>	This work
$\delta$ V3V5A10	ARO10 YEplac195- <i>PGK1p-ILV2</i> YEplac112- <i>PGK1p-ILV5</i>	
03-1AV2A10	W303-1A YEplac181- <i>TDH3p-Cox4-ARO10</i> YEplac195- <i>PGK1p-ILV2</i>	This work

Table 2 Plasmids used in this study

Plasmids	Description	Sources
YEplac181	<i>Amp<sup>r</sup> LEU2</i>	(Gietz <i>et al.</i> 1988)
YEplac195	<i>Amp<sup>r</sup> URA3</i>	(Gietz <i>et al.</i> 1988)
YEplac112	<i>Amp<sup>r</sup> TRP1</i>	(Gietz <i>et al.</i> 1988)
pUC18- <i>RYUR</i>	<i>Amp<sup>r</sup> URA3</i>	(Zhang <i>et al.</i> 2008)
YEplac181- <i>TDH3p-Cox4-ARO10</i>	<i>Amp<sup>r</sup> LEU2</i>	(Li, <i>et al.</i> 2017)
YEplac195- <i>PGK1p-ILV2</i>	<i>Amp<sup>r</sup> URA3</i>	(Li, <i>et al.</i> 2017)
YEplac112- <i>PGK1p-ILV5</i>	<i>Amp<sup>r</sup> TRP1</i>	This work
pUC18-5' $\delta$ - <i>PGK1p-ILV3-His3-63'</i>	<i>Amp<sup>r</sup> HIS3</i>	This work

Table 3 Primers used in this study.

Primer name	Primer sequences	Restriction site
<i>PGK1p-U</i>	5'GGGCCCGGATCCAGGCATTTGCAAGAATTACTC3'	<i>Bam</i> H $\square$
<i>PGK1p-D</i>	5'GGGCCCGTCCGACTGTTTTATATTTGTTGTAAA AAGTAG3'	<i>Sal</i> I
<i>ILV5ORF-U</i>	5'GGGCCCGTCCGACATGTTGAGAACTCAAGCCGC3'	<i>Sal</i> I
<i>ILV5ORF-D</i>	5'GGGCCCTGCAGACGTGACTTGACGAGTTG5'	<i>Pst</i> I
<i>ILV3 ORF-U</i>	5'GGGCCCGTCCGACATGGGCTTGTTAACGAAAGT3'	<i>Sal</i> I
<i>ILV3 ORF-D</i>	5'GGGCCCTGCAGGTAGAGGTGGCTTCGTCGTA3'	<i>Pst</i> I
<i>HIS3-U</i>	5'CGCGCTCTAGATTCGAAGAATATACTAAAA3'	<i>Xba</i> I
<i>HIS3-D</i>	5'CAGATGTCGACTCGAGTTCAAGAGAAAAAAA3'	<i>Sal</i> I
<i>Del1-U</i>	5'GGGCCCAAGCTTTGTTGGAATAGAAATCAACTATC3'	<i>Hind</i> III
<i>Del1-D</i>	5'GGGCCCGCATGCATGTTTATATTCATTGATC3'	<i>Sph</i> I
<i>Del2-U</i>	5'GGGCCCGAGCTCATAAAAATGATGATAATAAT3'	<i>Sac</i> I
<i>Del2-D</i>	5'GGGCCCGAATTCCTGAGAAATGGGTGAATGTTGAGAT3'	<i>Eco</i> R $\square$

Table 4 Comparison of strains' highest titers, yields and productivities of isobutanol and ethanol in VHGF fermentation.

Strains	Isobutanol titers (g/L)	Ethanol titers (g/L)	Isobutanol yield [mg/g glucose]	Ethanol yield [g/g glucose]	Isobutanol productivity (g/L/h)
Control	1.02±0.08	48.3±2.1	7.70±0.9	0.363±0.035	0.0213±0.0035
W303-1A	4.20±0.05	47.0±1.1	31.7±0.6	0.354±0.026	0.0876±0.0024
V26V3V5A10					
EMS39 V26V3V5A10	2.79±0.06	46.3±1.2	21.0±0.4	0.347±0.028	0.116±0.0030
EMS39	1.33±0.04	49.8±1.3	10.2±0.2	0.382±0.032	0.0416±0.0028
YEplac181YEplac195					
YEplac112					
HZAL-7 V26V3V5A10	3.46±0.07	45.2±1.4	26.2±0.7	0.343±0.019	0.0720±0.0019

## Figures

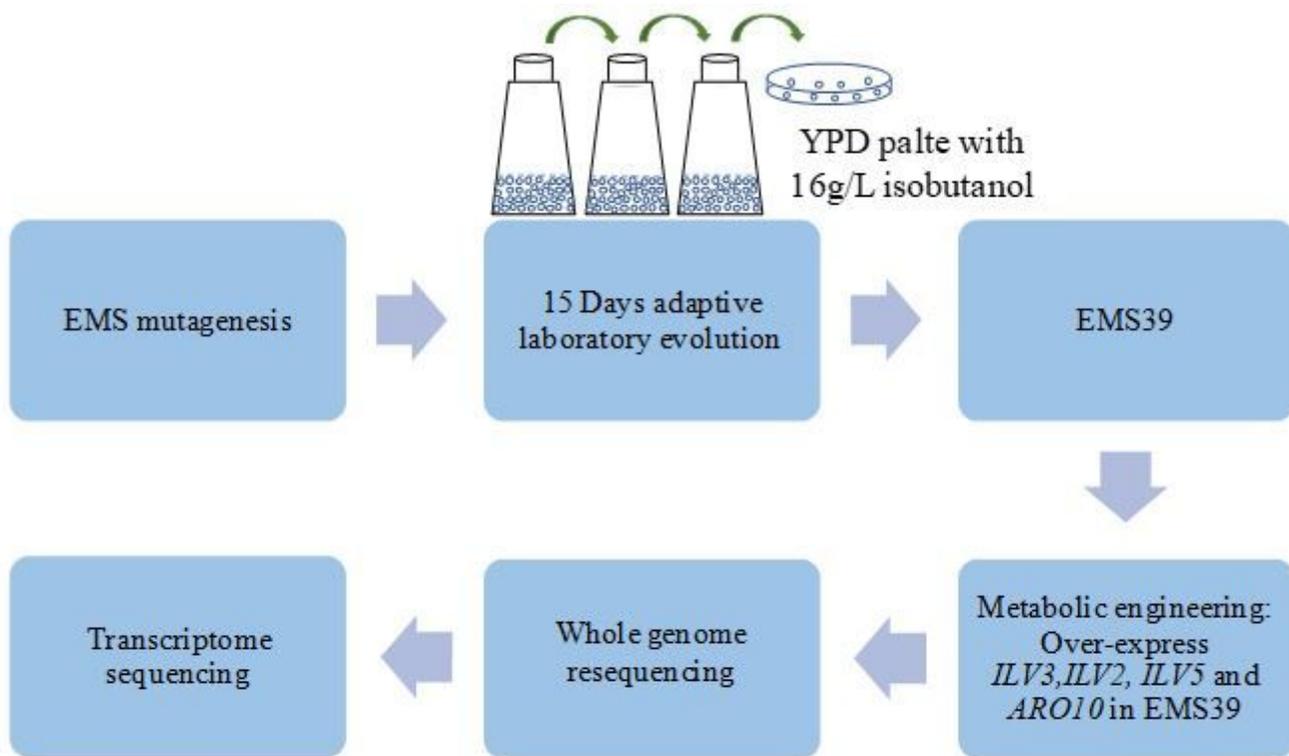


Fig.1

### Figure 1

Method for improving strains' isobutanol fermentation properties in very high-gravity fermentation through EMS mutagenesis and adaptive laboratory evolution in *Saccharomyces cerevisiae*. W303-1A was mutagenized by EMS, and the resulting population were subjected to 15 days Erlenmeyer flasks selection under higher glucose (100 g/L) and isobutanol (16 g/L) conditions throughout the cultivations. Then,  $\delta$ -

integration method was used to over-express ILV3 in the obtained mutant strains with desired phenotype. Meanwhile, ILV2, ILV5 and ARO10 were also over-expressed in the obtained mutant strains. Finally, the strain were tested for their isobutanol fermentation property.

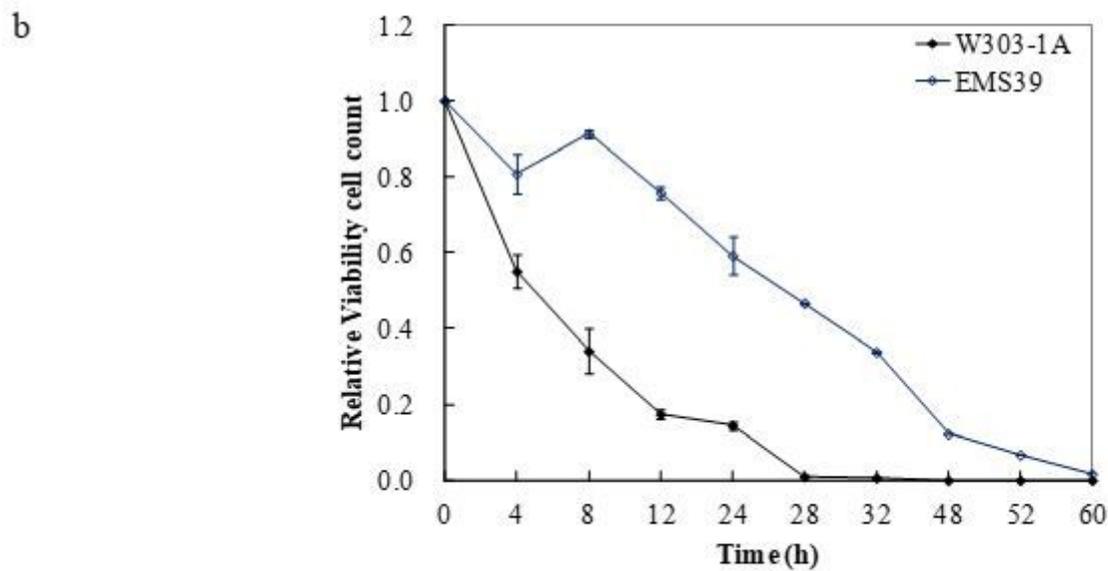
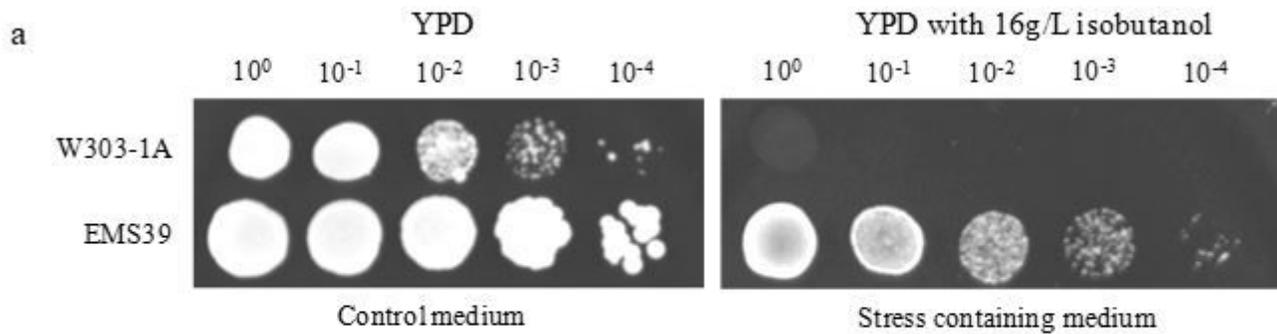


Fig. 2

## Figure 2

Isobutanol tolerance of EMS39 mutant obtained by EMS mutagenesis and adaptive laboratory evolution. (a) Spot assay test results of W303-1A and EMS39 population obtained by EMS mutagenesis and adaptive laboratory evolution on medium with and without 16 g/L isobutanol stress condition. Cells were spotted in 1:10 dilution series (from 10<sup>0</sup> to 10<sup>-4</sup>) on YPD plates without isobutanol (control medium) and with 16 g/L isobutanol containing medium. Growth was monitored upon 3 days of incubation. (b) The EMS39 mutant conferred a significantly enhanced viability. EMS39 mutant and the control (W303-1A) strain were treated and incubated in YPD medium in the presence of 20 g/L isobutanol. Viabilities of EMS39 mutant and the control (W303-1A) strain were measured as a function of time (hours) and expressed as the relative number of colony-forming units compared with colony count at 0 hours. Error bars represent the standard deviation between biological replicate experiments. Initial cell counts were  $\sim 3.5 \times 10^6$  cells per ml.

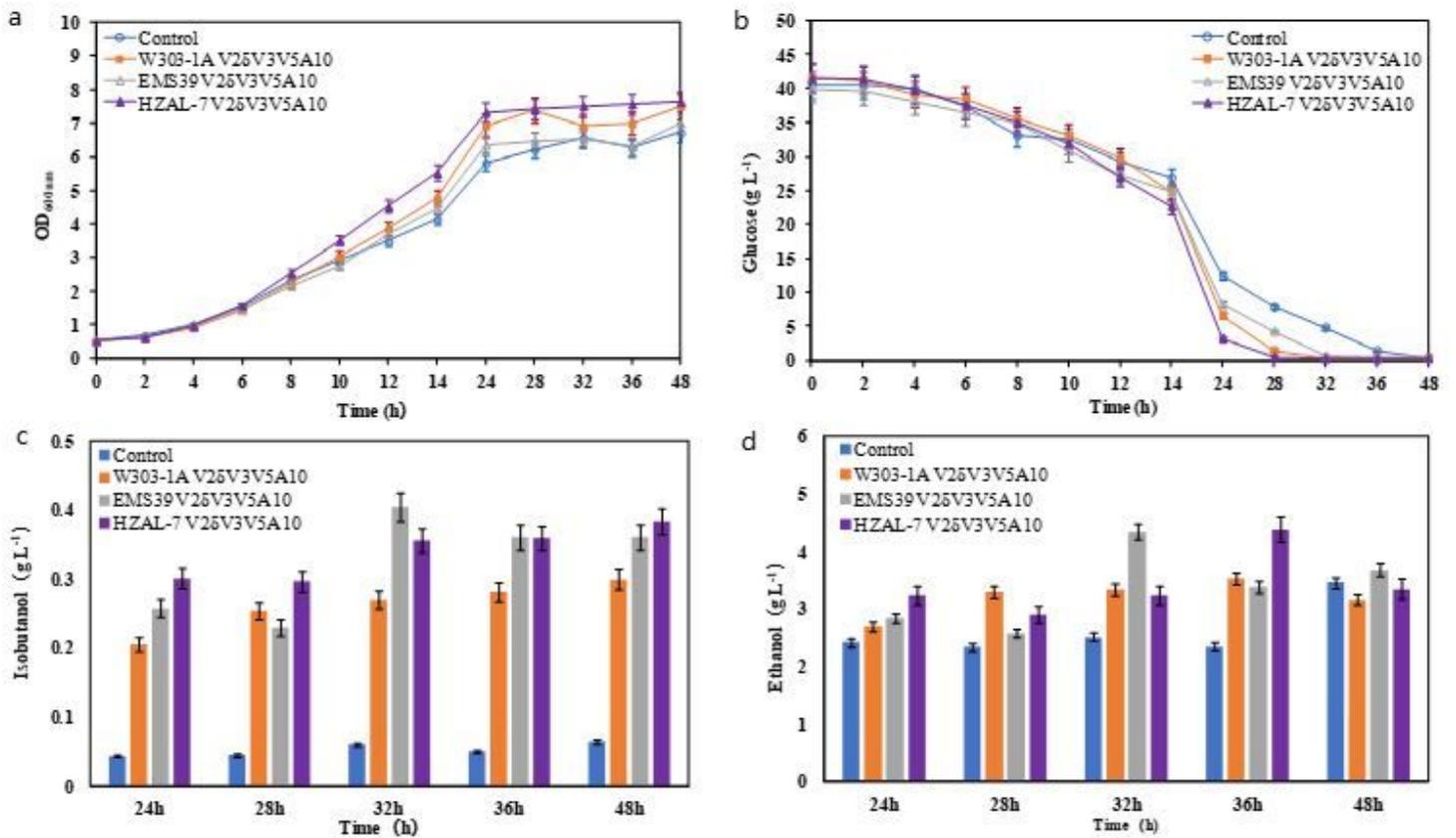


Fig.3

### Figure 3

Micro-aerobic batch fermentations (with 40 g/L initial glucose) of strains. Control, ●; W303-1A V2δV3V5A10, ■; EMS39 V2δV3V5A10, △; HZAL-7, ▲. (a) Growth curves, (b) Glucose consumption curves, (c) Isobutanol titers, (d) Production of ethanol. Data are presented as mean value and standard deviations of three independent biological replicates.

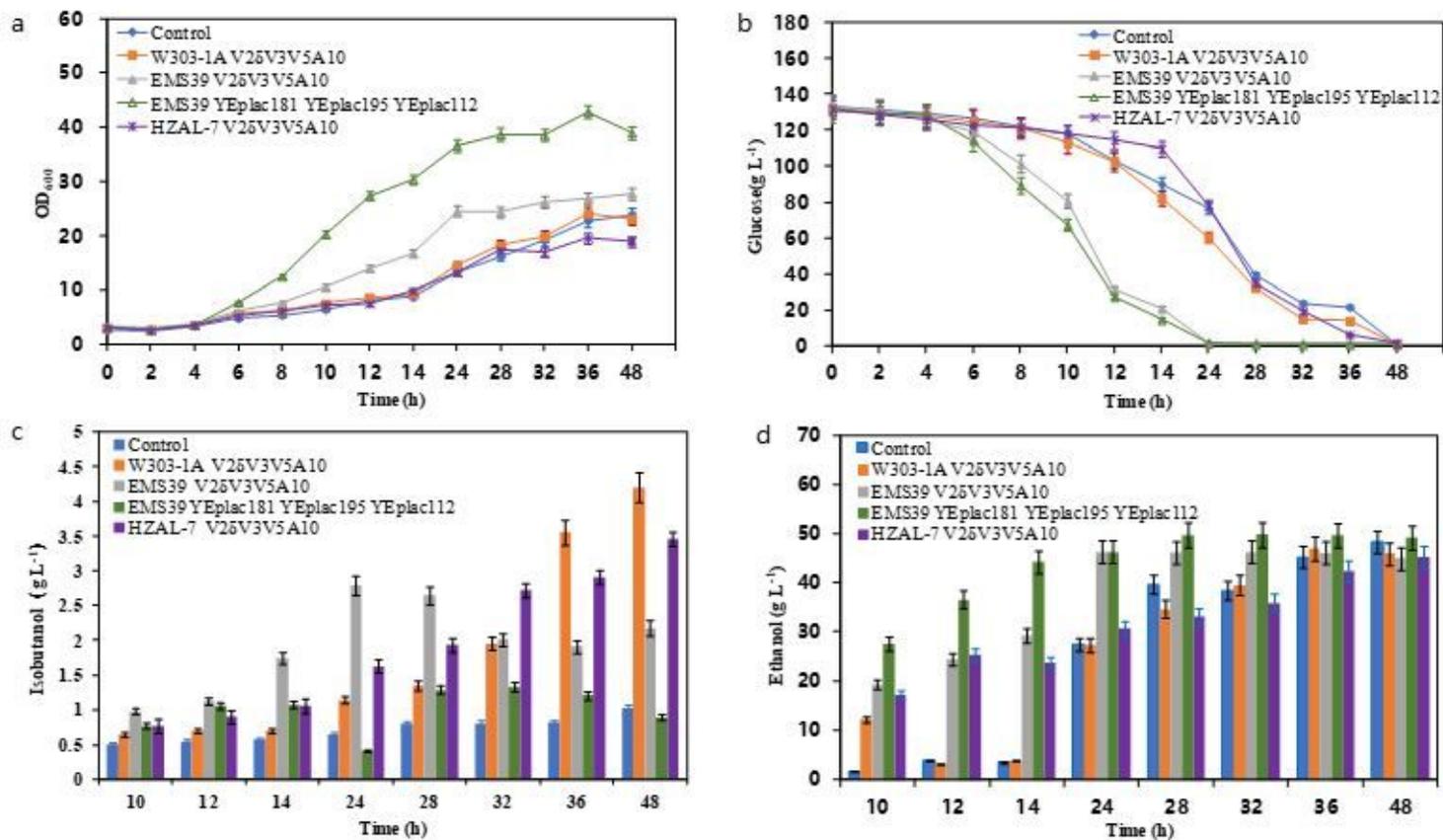


Fig.4

Figure 4

Very high-gravity fermentations (with 130 g/L initial glucose) of strains. Control, □; W303-1A V26V3V5A10, ■; EMS39 V26V3V5A10, ▲; EMS39 YEplac181 YEplac195 YEplac112, △; HZAL-7 V26V3V5A10, ▫. (a) Growth curves, (b) Glucose consumption curves, (c) Isobutanol titers, (d) Changes in ethanol titers. Data are presented as mean value and standard deviations of three independent biological replicates.

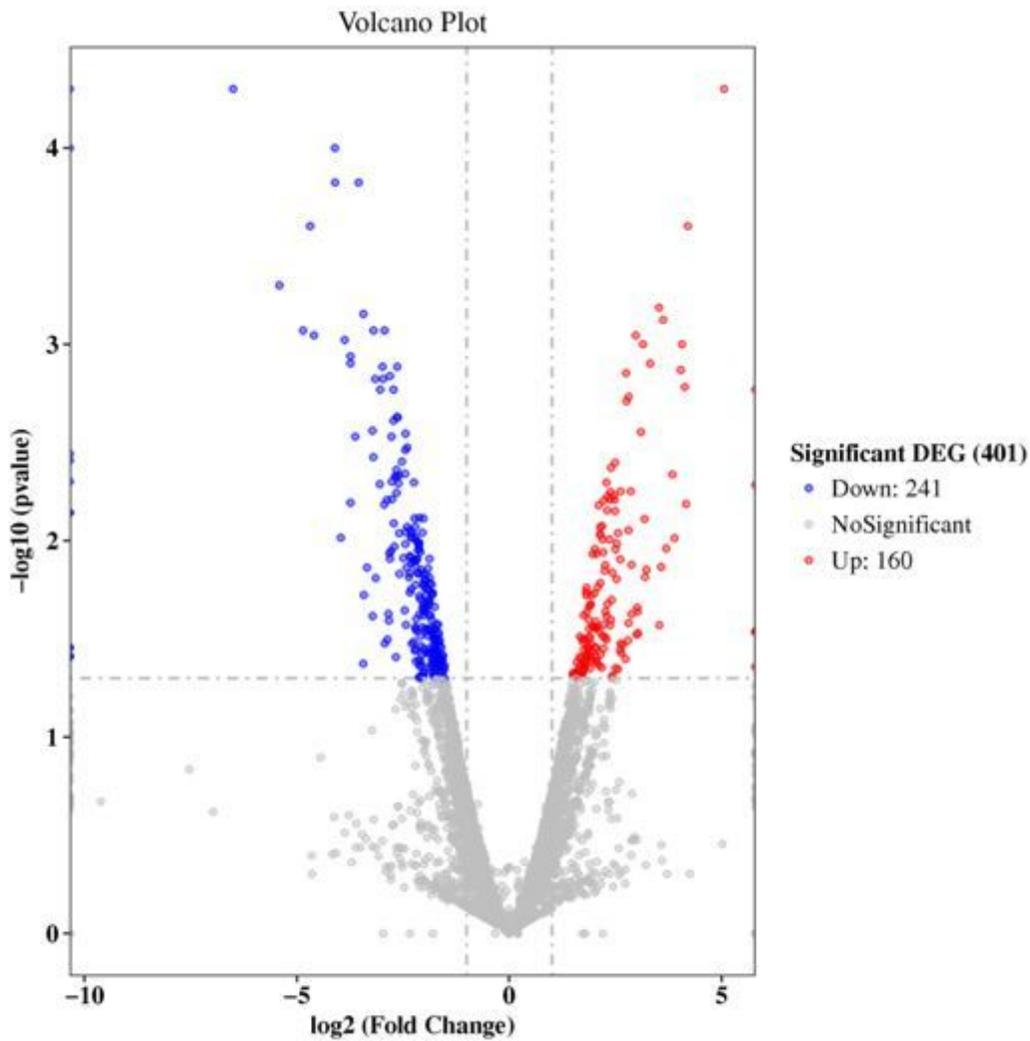


Fig.5

### Figure 5

Volcano plots of differentially expressed genes (DEGs) for W303-1Av3v2v5a10 VS EMS39v3v2v5a10. Genes with adjusted P values (Pvalues) less than 0.05 and log<sub>2</sub>(fold change) values greater than 1 were assigned as differentially expressed.



## Supplementary Files

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