Comparative Transcriptome and Metabolome Analyses Reveal the Methanol Dissimilation Pathway of *Pichia Pastoris*

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Research Article

Keywords: Transcriptome, Metabolomics, Yeast, Dissimilation pathway, Formaldehyde, DNA-protein crosslinking, Oxidative phosphorylation, Methanol metabolism, Proteasome, Glutathione

Posted Date: September 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-871571/v1>

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Abstract

Background: *Pichia pastoris* (*Komagataella phaffii*) is a model organism widely used for the recombinant expression of eukaryotic proteins, and it can metabolize methanol as its sole carbon and energy source. Methanol is oxidized to formaldehyde by alcohol oxidase (AOX), which is further metabolized either in the assimilation or dissimilation pathway. In the dissimilation pathway, formaldehyde is oxidized to CO₂ by formaldehyde dehydrogenase (FLD), S-hydroxymethyl glutathione hydrolase (FGH) and formate dehydrogenase (FDH). In addition, formaldehyde induces DNA-protein crosslinks (DPCs). Formaldehyde dehydrogenase is critical to minimize formaldehyde-mediated DNA lesions. Although phenotypes have been studied in engineered strains by modified dissimilation, there is a clear lack of systematic studies at the whole-omics level, especially transcriptomics and metabolomics.

Results: Focusing on the dissimilation pathway being cut off, we compared the transcriptomes and metabolomes from a formaldehyde dehydrogenase-deficient strain (Δfld), an S-hydroxymethyl glutathione dehydrogenase-deficient strain (Δfgh), a formate dehydrogenase deficient-strain (Δfdh) and the wild type (GS115). First, the differences between strains were most apparent after FLD knockout. When methanol was used as the sole carbon source, the differential metabolites between GS115 and Δfld were mainly enriched in ABC transporters, amino acid biosynthesis, and protein digestion and absorption. Second, analysis of differentially expressed genes (DEGs) between knockout and wild type strains under methanolic incubation showed that oxidative phosphorylation, glycolysis and the TCA cycle were downregulated, while proteasomes, autophagy and peroxisomes were upregulated. Transcription of alcohol metabolism was upregulated. It is worth noting that the degree of variation was positively correlated with the gene order of dissimilation pathway knockdown. In addition, there were significant differences in amino acid metabolism and glutathione redox cycling that raised our concerns about formaldehyde sorption in cells.

Conclusions: This is the first time that integrity of dissimilation pathway analysis was carried out in *Pichia pastoris* on the basis of transcriptomics and metabolomics. Truncation of the dissimilation pathway affected methanol metabolism, and knockdown of FLD impaired formaldehyde assimilation. The significant downregulation of oxidative phosphorylation may reveal that FLD and FGH are key enzymes in the energy utilization of cellular methanol metabolism. In addition, formaldehyde can not only bind glutathione but also react with amino acids, especially cysteine. The upregulation of the proteasome and autophagy may solve the problem of DNA-protein crosslinking caused by formaldehyde.

Background

The methylotrophic yeast *Pichia pastoris* (*Komagataella phaffii*) is one of the most commonly used expression systems. It can metabolize methanol as its sole carbon and energy source [1–3]. The methanol metabolism in *Pichia pastoris* is summarized in Fig. 1. Formaldehyde is further metabolized by assimilation or dissimilation. Methanol is oxidized to formaldehyde by alcohol oxidase (AOX) in peroxisomes. During assimilation, formaldehyde is directly fixed with dihydroxyacetone synthase (DAS) [4]. The entire methanol assimilation pathway is localized to peroxisomes. In the dissimilation pathway, formaldehyde is oxidized to S-formylglutathione by the NAD⁺-dependent enzyme formaldehyde dehydrogenase (FLD) in the first step, and S-formylglutathione then reacts spontaneously with glutathione to form S-hydroxymethyl glutathione. Then, S-formylglutathione hydrolase (FGH) hydrolase hydrolyses this compound into formate and glutathione. Next, formate is oxidized to CO₂ by NAD⁺-dependent formate dehydrogenase (FDH) and released in vitro [5–8].

The dissimilation pathway metabolizes 2 molecules of NADH to the extent that it is considered to be the main source of power and energy. A surplus of FLD activity was shown to result in higher theoretical NADH formation rates and finally also in significantly improved butanediol production rates when a *P. pastoris* strain overexpressing FLD and butanediol dehydrogenase was applied for whole-cell biotransformation [9]. Additionally, by deleting the genes coding for dihydroxyacetone synthase isoforms 1 and 2 (DAS1 and DAS2), NADH regeneration via methanol oxidation (dissimilation) was increased significantly, which led to an increase in ATP and higher S-adenosylmethionine production [8, 10]. Therefore, an enhanced dissimilation pathway optimizes the energy distributions of methanol metabolism, guaranteeing more efficient NADH/product coupling and exploitation of both NADH steps for cofactor regeneration in whole-cell biotransformations [9].

Unfortunately, a carbon mass balance analysis revealed that 70–80% of the methanol metabolized is converted into carbon dioxide in the methanol fed-batch phase, when the methanol feed is in excess of the metabolic requirements in general [11–13]. This involves a huge loss of carbon atoms. Previous studies have reported the prevention of the conversion of methanol to CO₂ via formaldehyde and formate by the knockout of genes for enzymes related to the dissimilation pathway. However, the Δfld strain suffered severe growth defects upon the addition of formaldehyde [14]. Similarly, whether in the methanol induction stage or the subsequent fermentation stage, the Δfdh strain showed a lower biomass and a slightly slower methanol consumption rate [15]. Knockout studies on FLD and FDH of methylotrophic yeast demonstrated that the FLD knockout phenotype is more severe than the FDH knockout phenotype, which was explained by the higher toxicity of formaldehyde compared to formate [14]. Simply knocking out or weakening one or more genes in the dissimilation pathway without making up for the loss of NADH and ATP, not only results in the accumulation of toxic intermediates but also causes energy imbalance. It is instructive to determine the

role of the dissimilation pathway before balancing carbon flow distribution and energy supply in the production and application of engineered yeast ^[15, 16].

The growth defect of methylotrophic yeast on high methanol medium is not caused directly by methanol toxicity but rather by formaldehyde, which is a key toxic intermediate of methanol metabolism ^[17]. The toxicity of formaldehyde leads to partial synthetic lethality and growth defects in various cell lines utilizing methanol as a carbon source. Similarly, mice deficient in formaldehyde-metabolizing enzymes were found to develop partial synthetic lethality and mortality shortly after birth. This phenotype may be due to the accumulation of endogenous formaldehyde ^[18]. In fungi, plants or animals, the induction of DNA-protein crosslinking (DPC) formaldehyde is a topic of serious interest. As a strongly polarized reactive carbonyl compound, formaldehyde exists in various parts of the organism and plays an important role in cognitive ability and memory formation ^[19]. Considering that DNA repair and RNA degradation pathways are evolutionarily conserved from yeast to humans, mechanisms of formaldehyde toxicity identified in yeast may be relevant to human disease and genetic susceptibility ^[20]. Formaldehyde dehydrogenase is critical to minimize the DPC issue, and gene knockout organisms are commonly used to study the intracellular pathological changes of formaldehyde ^[18, 21, 22]. DPCs mainly consist of ribosomes and outer membrane proteins. Malfunction of these proteins may cause cell death because of outer membrane porin-induced programmed cell death or metabolic flux imbalance ^[23]. Membrane structure is a major target of methanol toxicity, while proteins are major targets of formaldehyde toxicity ^[24].

The dissimilation pathway is speculated to have two physiological functions: formaldehyde detoxification and energy production ^[4, 25, 26]. Based on these two points, we constructed three dissimilation pathway single gene knockout strains by CRISPR/Cas9: a formaldehyde dehydrogenase-deficient strain (Δfld), an S-(hydroxymethyl) glutathione dehydrogenase-deficient strain (Δfgh) and a formate dehydrogenase-deficient strain (Δfdh). Then, we analysed key nodes and metabolic pathways by examining differentially expressed genes (DEGs) to discover the function of the dissimilation pathway and explore the stress response of yeast by comparing the transcriptome and metabolome of dissimilation pathway-defective strains and wild-type strains. Performing a whole-transcriptome analysis of dissimilation pathway knockout can promote the pathological study of formaldehyde metabolism and the development of industrial production strains.

Results And Discussion

Phenotypic and quantitative genetic analysis

We knocked out enzyme genes of the dissimilation pathway (FLD (PAS_chr3_1028), FGH (PAS_chr3_0867) and FDH (PAS_chr3_0932)) separately in GS115 by CRISPR/Cas9 and found no homologous sequences in the genome through BLAST (Fig. 1) ^[27]. Glucose was used as the main carbon source, and there was no obvious growth difference among strains. Methanol was used as the main carbon source, and the growth of dissimilation pathway knockout strains was worse than that of wild-type strain (GS115) (Fig. 2. A). We conducted pairwise transcriptome comparisons between wild-type and dissimilation pathway knockout strains after culture for 12 hours with glucose and 1% methanol as the main carbon sources. Sample clusters with high similarity between the strains under glucose culture conditions were identified. The samples of strains under methanol culture conditions were significantly different. The transcriptional profiles of Δfld , Δfgh , Δfdh and GS115 were developed and designated KO_FLD, KO_FGH, KO_FDH and GS115 respectively. We found that outlier samples (KO_FLD) showed the lowest correlation coefficient, which means a less similar gene expression level. GS115 and KO_FDH have the smallest sample variation.

DEGs between wild-type and dissimilation pathway knockout groups

Transcriptome comparisons were designed to reflect the effects of metabolic pathways induced by gene knockout or methanol perturbations between dissimilation pathway knockout and wild-type strains in three groups: GS115 versus KO_FLD (GL), GS115 versus KO_FGH (GG) and GS115 versus KO_FDH (GD). The results of the significantly DEGs detected, based on the gene expression levels of individual samples, are as follows (Fig. 2. B). The order of the knockout affected the level of transcription. Screening for highly expressed genes ($\log_2FC \geq 2$, $q \leq 0.05$) in GL revealed the upregulation of some dehydrogenase transcripts after FLD knockout, such as pyridoxine 4-dehydrogenase (PAS_chr4_0550), alcohol dehydrogenase (NADP+) (PAS_chr3_0006), NADPH2 dehydrogenase (PAS_chr3_1184), D-arabinose 1-dehydrogenase (PAS_chr2-1_0775), which may imply that other dehydrogenases compensate for the function of formaldehyde dehydrogenase, in GG, ubiquitin C (PAS_chr4_0762), AN1-type domain-containing protein (PAS_chr4_0567), 20S proteasome subunit alpha 2 (PAS_chr1-1_0433), which is in ubiquitin-proteasome pathway, was significantly upregulated, in GD the difference was not significant.

To understand the common impact of knockouts in the dissimilation pathway, we used Venn diagrams to show genes in different comparison groups. A total of 137 DEGs among the three groups were enriched in KEGG metabolic pathways ($\log_2FC \geq 0.5$, $q \leq 0.05$) (Fig. 2. C): glycolysis, TCA cycle, pentose and glucuronate interconversions, pyruvate metabolism biosynthesis of antibiotics, metabolism of various amino acids (tyrosine, phenylalanine, tyrosine and tryptophan, glycine, serine and threonine). A total of 137 DEGs among GL and GG were enriched in KEGG metabolic pathways ($\log_2FC \geq 1$, $q \leq 0.05$) (Fig. 2. C): most genes in the peroxisome pathway were transcriptionally upregulated, while oxidative

phosphorylation, glycolysis and TCA cycle harbored a large proportion of genes that were significantly downregulated. Furthermore, we were interested in genes involved in cysteine metabolism ($p \leq 0.05$) and glutathione metabolism due to formaldehyde binding.

Comparison transcriptome of Δfld and Wild-type strain

Formaldehyde dehydrogenase is the first enzyme in the dissimilation pathway, and its knockout can severely compromise strain robustness. We used BMM medium with methanol as the sole carbon source for 24 h to evaluate the metabolomic differences between Δfld and wild-type strain.

All identified metabolites were clustered according to chemical classification, and the proportions of each type of metabolite are shown (Fig. 2. D): organic acids and derivatives (32.561%), (lipids and lipid-like molecules (19.562%), and organoheterocyclic compounds (12.227%). Based on univariate analysis, the differences among all metabolites (including unidentified metabolites) detected in positive and negative ion modes were analysed. There were significantly increased nucleosides, nucleotides, analogues, and organic oxygen compounds in positive ion mode and organic heterocyclic compounds, organic nitrogen compounds and benzenoids in negative ion mode. DL-threonine, meperidine, L-homoserine, and several organic acids and derivatives were reduced. A comparative analysis of differences between GL showed 138 differential metabolic pathways, with the top three significantly enriched differential metabolic pathways being ABC transporters, amino acid biosynthesis, and protein digestion and absorption (Additional Figure. 1).

Methanol metabolism pathway

The main carbon metabolism pathways in *P. pastoris* include methanol metabolism, glycolysis, the TCA cycle, the pentose phosphate pathway and ethanol metabolism. By comparing transcriptomes, we attempted to explain the variation in the methanol metabolism pathway after knockout of the heterotrimeric pathway genes (Fig. 1).

Previous studies have shown that under methanol culture conditions, genes encoding methanol metabolism are upregulated, and glycolysis and TCA cycle transcription are downregulated [28]. Significant transcriptional downregulation of genes involved in glycolysis and the TCA cycle was observed in GL and GG. Interestingly, malate dehydrogenase (MDH2, PAS_chr4_0815) was transcriptionally upregulated in GL, GG ($\log_2FC \geq 2.7$, $q \leq 7.93E-06$) and GD. Genes involved in alcohol metabolism were significantly transcriptionally upregulated ($q \leq 0.05$). Among them, acetyl-coenzyme A synthetase transcription (ACAS1, PAS_chr3_0403) and alcohol dehydrogenase (ADH, PAS_chr1-3_0153) transcription were upregulated ($\log_2FC \geq 1$, $q \leq 0.05$). Aldehyde dehydrogenase (NAD⁺) (ALDH, PAS_chr3_0987) ($\log_2FC \geq 3.7$, $q \leq 6.61E-07$) was significantly upregulated in GG. However, ACAS2 (PAS_chr2-1_0767) was downregulated in GL and GG ($\log_2FC \leq -1.4$, $q \leq 6.61E-07$). This may mean that the two ACASs are responsible for different metabolic pathways in *Pichia pastoris* and that ACAS2 is more affected by methanol induction.

With the exception of FLD, knockdown of FGH and FDH genes had little effect on the assimilation and dissimilation pathways of methanol. In contrast, the dihydroxyacetone synthase (DAS, PAS_chr3_0832 and PAS_chr3_0834, $\log_2FC \leq -0.8$) and fructose-bisphosphate aldolase (FBA, PAS_chr1-1_0072, $\log_2FC \leq -1.7$) of the assimilation pathway were significantly downregulated when FLD was knocked out, which may be one of the reasons for the low biomass of KO_FLD. This again validates the prominence of FLD in the dissimilation pathway. The FLD1 promoter is strongly and independently induced by either methanol as the sole carbon source (with ammonium sulfate as the nitrogen source) or methylamine as the sole nitrogen source (with glucose as the carbon source). FLD coordinates the formaldehyde level in methanol-grown cells according to the methanol concentration on growth [29]. Furthermore, in peroxisomes, alcohol oxidase 2 (AOX2, PAS_chr4_0152) was upregulated in GL ($\log_2FC \geq 1.3$, $q \leq 4.96E-12$) and GD ($\log_2FC \geq 0.9$, $q \leq 1E-04$). AOX2 is strongly induced by methanol, which may lead to an increase in formaldehyde content in the peroxisome.

When FGH was knocked out, catalase (CAT, PAS_chr2-2_0131, $\log_2FC \geq 1$) was upregulated, which means increased oxidative stress. Glutathione peroxidase (GPX, PAS_chr2-1_0033, $\log_2FC \geq 0.6$) was upregulated, and glutathione reductase (GSR, PAS_chr3_1011, $\log_2FC \leq -0.8$) was downregulated. The distribution of FGH between peroxisomes and the cytosol was demonstrated [30]. Knockdown of FGH may affect the binding of formaldehyde to glutathione.

Oxidative phosphorylation

The majority of methanol is metabolized via an energy-generating dissimilation pathway, leading to a corresponding increase in mitochondrial size and number [12]. The 60 DEGs from the three groups in the oxidative phosphorylation pathways were clustered, and the GL and GG genes were significantly downregulated (Fig. 3). The oxidative phosphorylation of the wild type was equivalent to that of KO_FDHD. The physiological role of FDH was revealed to be mainly detoxification of formate rather than stimulated energy generation [31]. In GD, only NADH dehydrogenase (NDH, PAS_chr3_0792), a mitochondrial external NADH dehydrogenase or type II NAD(P)H: quinone oxidoreductase, was upregulated ($\log_2FC \geq 1.2$, $q \leq 0.01$). It may make up for the lack of FDH.

Our transcripts validate the importance of FLD for energy supply. FLD knockout reduces NADP supply. Therefore, FLD activity was identified as the main bottleneck of effective recovery of NADH through methanol dissimilation. In the engineered strain modification, butanediol productivity was improved by increasing FLD activity [9]. The same was true for the knockout of FGH. However, the knockout of FGH affected the recovery of GSH. In GG, cytochrome c oxidase subunit 7c (COX7C, PAS_chr2-2_0266) ($\log_2FC \geq 2.9$, $q \leq 0.01$) and haem o synthase (COX10, PAS_chr1-3_0194) ($\log_2FC \geq 2.0$, $q \leq 0.01$) were significantly upregulated. However, downregulation of other genes affects ATP production.

Adsorption of formaldehyde by glutathione and amino acids

The methanol metabolism-related generation of reactive oxygen species (ROS) induced a pronounced oxidative stress response. CAT upregulation after FGH knockdown increased ROS. Methanol oxidation stress results in the accumulation of formaldehyde and hydrogen peroxide [32, 33]. Superoxide dismutase [Cu-Zn] (PAS_chr4_0786), which destroys radicals normally produced within cells that are toxic to biological systems, was upregulated significantly in GL ($\log_2FC \geq 1.0$, $q \leq 0.01$) and GG ($\log_2FC \geq 2.5$, $q \leq 0.01$).

Glutathione (GSH, L- γ -glutamyl-L-cysteinylglycine) is the main sulfur compound and appears as the major nonprotein thiol compound in yeasts. An important reaction by formaldehyde is the formation of compounds with the tripeptide glutathione, between 50% and 80% of endogenous formaldehyde occurs in the form of compounds that include glutathione [16, 34]. GSH scavenges cytotoxic H_2O_2 and maintains a redox balance in the cellular compartments [35]. GSH seems to be involved in the response of yeasts to different nutritional and oxidative stresses [36]. The highest overall glutathione levels correlated with their high viability. In cells, glutathione mainly exists in the reduced form GSH, as oxidized glutathione (GSSG) is converted rapidly by glutathione reductase. GSH/GSSG ratio rose, suggesting stronger protection against oxidative stress, and was also correlated with high glutathione reductase activity [37, 38]. In the process of glutathione reduction and oxidation, glutathione peroxidase (GPX, PAS_chr2-2_0382) was upregulated ($\log_2FC \geq 0.6$, $q \leq 0.05$), while glutathione reductase (NADPH) (GSR, PAS_chr3_1011) was downregulated ($\log_2FC \leq -0.6$, $q \leq 0.05$) in GG. In the metabolome, knockdown of fld increased the amount of GSSG compared to that in the original strain. Knockdown of FLD and FGH may accelerate the GSH redox cycle.

The formation of S-[1-(N2-deoxyguanosinyl) methyl] glutathione between glutathione and DNA was induced by formaldehyde. The involvement of the cysteine residue of GSH in coupling suggests that other thiols may participate in the formation of this type of DNA damage from formaldehyde [39]. Formaldehyde also reacts easily with proteins and decreases the number of free amino groups. When mammalian cells are exposed to formaldehyde, the levels of the reaction products of formaldehyde with the amino acids cysteine and histidine, namely, timonacic and spinacine, are increased. These reactions take place spontaneously, and the formation of timonacic is reversible. The reactions of formaldehyde with cysteine and histidine are alternative routes of formaldehyde metabolism [40]. Methanol-grown cells have a higher protein content but lower free amino acid content. In the context of the upregulation of many amino acid biosynthesis genes or proteins, this suggests an increased flux towards amino acid and protein synthesis, which is also reflected in increased levels of transcripts and/or proteins related to ribosome biogenesis and translation [41]. With FLD knockout, genes for ribosomal and amino acid metabolism are downregulated, affecting amino acid synthesis and thus weakening growth.

We clustered the 149 DEGs in the amino acid metabolism. 5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (metE, PAS_chr2-1_0160) had zero expression in KO_FLD and was upregulated in GG ($\log_2FC \geq 1.6$, $q \leq 0.05$). Cystathionine gamma-lyase (CTH, PAS_chr1-4_0489) had zero expression in GS115_M. CTH, an enzyme involved in sulfur compound metabolism and cysteine metabolism, showed significantly upregulated transcription ($\log_2FC \geq 10$, $q \leq 2.7E-54$). This may verify the possible adsorption of formaldehyde by glutathione and amino acids. Afterwards, we performed a differential clustering analysis using the remaining 147 genes (Additional Figure. 2). The knockout of each dissimilation gene produced a comparable up- and down-regulation trend, which may reveal the effect of knocking out the dissimilation pathway on the amino acid pathway. GO enrichment analysis revealed that in GL and GG, genes involved in the metabolic and biosynthetic processes of organic acids and carboxylic acids were transcriptionally downregulated, whereas in GD, they were transcriptionally upregulated ($q \leq 0.01$). Another difference is that primary amine oxidase (AOC3, PAS_chr1-4_0441, PAS_chr2-1_0307 and PAS_chr4_0621) and amidase (amiE, PAS_chr3_0283) are upregulated in GL and GD. The knockout of two dehydrogenases in the dissimilation pathway may have affected the metabolism of organic nitrogen compounds.

Proline dehydrogenase (PRODH, PAS_chr1-3_0269) is upregulated in order of knockout by sequence ($\log_2FC \geq 1.4, 2.8, 3.4$, $q \leq 0.01$), which facilitates the process of proline catabolism to glutamate. The glutamate-cysteine ligase catalytic subunit (GCLC, PAS_chr1-1_0184) is transcriptionally upregulated in GG and GD ($\log_2FC \geq 0.5$, $q \leq 0.01$). Glutathione S-transferase (GST, PAS_chr2-1_0490) was significantly upregulated in GG ($\log_2FC \geq 1.6$, $q \leq 0.01$) and downregulated in GL ($\log_2FC \leq -1.3$, $q \leq 0.05$). Cys-Gly metallodipeptidase (DUG1, PAS_chr3_0353) was significantly upregulated in GL ($\log_2FC \geq 2.1$, $q \leq 1.5E-07$).

Effect of DNA cross-linking on proteasome and peroxisome

Excessive ROS are formed from peroxisome metabolism when methanol-grown wild-type cells are exposed to excess methanol [42]. Upregulation of the peroxisome in the dissimilation pathway knockout strains pathway may be closely related to the concentration of formaldehyde. The upregulation of the autophagy pathway during the methanol induction phase may be related to the degradation of damaged peroxisomes [43]. Accumulating evidence indicates that damaged components of eukaryotic cells are removed by autophagic degradation [44]. The knockdown of genes involved in the dissimilation pathway caused upregulation of peroxisomes and autophagy.

Mutations in the gene encoding the putative human homologue of a yeast DPC protease cause premature ageing and cancer predisposition syndrome in humans [45]. Proteolytic cleavage of the protein moiety of a DPC is a general strategy for removing the lesion and can be accomplished through a DPC-specific protease and/or proteasome-mediated degradation. Nucleotide excision repair and homologous recombination are each involved in repairing DPCs, with their respective roles likely dependent on the nature and size of the adduct [46]. The transcription level of the DNA-dependent metalloprotease WSS1 (PAS_chr3_Q200) increased significantly ($\log_2FC \geq 1.1$ (GG), $\log_2FC \geq 1.9$ (GL), $q \leq 0.01$), and the proteasome gene was significantly upregulated after the dissimilation pathway was knocked out in methanol culture. This indicated that protease digestion of DPC was a stress response to formaldehyde. The protein component of DPCs is targeted for repair by proteases of the Wss1/SPRTN family. Formaldehyde exposure triggers widespread ubiquitylation events in cells [47, 48]. The proteasome may eliminate DNA conjugates caused by excessive formaldehyde. The proteasome is a large protein complex responsible for the degradation of intracellular proteins. The polymerization of ubiquitin, a key molecule known to work in concert with the proteasome, serves as a degradation signal for numerous target proteins, the destruction of a protein is initiated by covalent attachment of a chain consisting of several copies of ubiquitin. In eukaryotes, the autophagy–lysosome system and the ubiquitin–proteasome system (UPS) are the two major quality control pathways responsible for maintaining proteome homeostasis and directing recycling to meet nutrient demand. In contrast, autophagy can eliminate larger protein complexes, insoluble protein aggregates, and even entire organelles and pathogens in toto due to the sheer size of the engulfing autophagic vesicles [49]. The ubiquitin–proteasome system controls almost all basic cellular processes—such as progression through the cell cycle, signal transduction, cell death, immune responses, metabolism, protein quality control and development-by degrading short-lived regulatory or structurally aberrant proteins, connecting ubiquitylation and autophagy as key regulatory events in proteasome quality control [49, 50]. In *Saccharomyces cerevisiae*, several genes involved in DNA repair (eight RAD genes) that have been identified as specific for methanol toxicity were previously reported as determinants of tolerance for formaldehyde, a methanol detoxification pathway intermediate [51]. Knockdown of FLD and FGH allows the accumulation of formaldehyde, leading to upregulation of DNA repair genes. It is also of interest that genes related to DPCs in *P. pastoris* could be mining.

Conclusions

Knockdown of the dissimilation pathway leads to downregulation of glycolysis and the TCA cycle. In particular, the assimilation pathway was downregulated when FLD was absent. Knockdown of FLD and FGH significantly downregulated oxidative phosphorylation, leaving the strains with inadequate energy supply and low biomass. Differences in amino acid and glutathione metabolism were apparent, probably due to the adsorption of cysteine and glutathione to formaldehyde to resolve formaldehyde accumulation and oxidative stress responses. In addition, knockdown of the dissimilation pathway enhanced the stress response to formaldehyde. Upregulation of the proteasome, peroxisome and autophagy may be the solution to formaldehyde-induced DPC.

Methods

Strains and cultivation

P. pastoris GS115 (Invitrogen, USA), a histidine auxotrophic strain, was used as the host strain for the construction of recombinant strains [52]. Methylophilic yeast grows well in a liquid medium containing 1% (vol/vol) methanol as the carbon source to induce the production of heterologous proteins. Culture conditions for transcriptome analysis: the strains were grown on YPD agar medium containing 2% agar, 1% yeast extract, 2% peptone and 2% glucose to obtain the seed liquid. In YP liquid medium (1% yeast extract, 2% peptone) containing 1% (vol/vol) methanol (YPM medium) or 2% glucose (YPD medium), the cells were cultured for 12 h to the exponential logarithmic phase of growth [53]. Culture conditions for metabolome analysis were as follows: in BMM liquid medium containing 100 mM potassium phosphate (pH 6.0), 1.34% YNB, 4×10⁻⁵% biotin and 0.5% methanol, the cells were cultured for 24 h.

CRISPR/Cas9

P. pastoris GS115 was used as the host for CRISPR/Cas9-based genome editing studies. Efficient CRISPR/Cas9-mediated genome editing with a type III promoter (i.e., SER promoter) to drive the expression of the single guide RNA (sgRNA) was achieved [54]. Using CRISPRdirect (<http://crispr.dbcls.jp/>) to design a targeting sequence, a CRISPR plasmid vector (Cas9-III promoter-targeting sequence-gRNA) was successfully constructed [55]. The donor was synthesized with the upper and lower 1000 bp of the target gene. Donor and Cas9 plasmids were transformed

into *Pichia pastoris* for expression (100 µg/mL ampicillin supplemented when necessary). The successful knockout strains were screened and cultured in YPD for a period of time to remove the plasmids.

Total RNA extraction

Fungi RNA was extracted using the TRIzol® method following the manufacturer's protocol. Bulk cells or filamentous fungus are grounded to a powder using liquid nitrogen, and the powder was transferred into the 2ml tube contains 1.5mL Trizol reagent. The mix was shaken for 3min, and kept for 5min at room temperature, then was centrifuged at 10,000×g for 5min at 4°C. The supernatant was added 200µL of chloroform/isoamyl alcohol (24:1) with 1mL lysis reagents. After centrifuged at 10,000×g for 10min at 4°C, the supernatant was transferred into another new tube with equal volume of isopropanol and put in the refrigerator at -20°C for 1h. After centrifuged at 13600×g for 20min at 4°C, the supernatant was precipitated by 1 mL of 75% ethanol and let dry for 3-5min. The RNA pellet was dissolved with 30-100µL of DEPC water or RNase-free water. The concentration of the extracted RNA samples was determined using a Nanodrop system (NanoDrop, Madison, USA), and the integrity of the RNA was examined by the RNA integrity number (RIN) using an Agilent 2100 bioanalyzer (Agilent, Santa Clara, USA).

mRNA Library Construction

DNase I was used to digest double-stranded and single-stranded DNA in total RNA, then, magnetic beads were purified to recover the reaction products, RNase H or Ribo-Zero method (human, mouse, plants) (Illumina, USA) was used to remove the rRNA. Purified mRNA from previous steps was fragmented into small pieces with fragment buffer at appropriate temperature. Then, First-strand cDNA was generated in First Strand Reaction System by PCR, and the second-strand cDNA was generated as well. The reaction product was purified by magnetic beads, afterwards, A-Tailing Mix and RNA Index Adapters were added by incubating to carry out end repair. The cDNA fragments with adapters were amplified by PCR, and the products were purified by Ampure XP Beads. Library was validating on the Agilent Technologies 2100 bioanalyzer for quality control. The double stranded PCR products above were heated denatured and circularized by the splint oligo sequence. The single strand circle DNA (ssCir DNA) was formatted as the final library. The final library was amplified with phi29 (Thermo Fisher Scientific, MA, USA) to make DNA nanoball (DNB) which had more than 300 copies of one molecular, DNBs were loaded into the patterned nanoarray and single end 50 bases reads were generated on BGISEQ500 platform (BGI-Shenzhen, China).

Transcriptome

The sequencing data was filtered with SOAPnuke (v1.5.2) by (1) Removing reads containing sequencing adapter, (2) Removing reads whose low-quality base ratio (base quality less than or equal to 5) is more than 20%, (3) Removing reads whose unknown base (N' base) ratio is more than 5%, afterwards clean reads were obtained and stored in FASTQ format. The clean reads were mapped to the reference genome using HISAT2 (v2.0.4). Bowtie2 (v2.2.5) was applied to align the clean reads to the reference coding gene set, then expression level of gene was calculated by RSEM (v1.2.12). The heatmap was drawn by pheatmap (v1.0.8) according to the gene expression in different samples. Essentially, differential expression analysis was performed using the DESeq2 (v1.4.5) with Q value ≤ 0.05 . To take insight to the change of phenotype, GO (<http://www.geneontology.org>) and KEGG (<https://www.kegg.jp/>) enrichment analysis of annotated different expressed gene was performed by Phyper (https://en.wikipedia.org/wiki/Hypergeometric_distribution) based on Hypergeometric test. The significant levels of terms and pathways were corrected by Q value with a rigorous threshold (Q value ≤ 0.05) by Bonferroni [56-62].

Metabonomics

In this experiment, the metabolic profile changes of the samples were analysed by a metabonomics method based on UHPLC-Q-TOFMS technology. Metabonomics usually takes strict OPLS-DA VIP>1 and P value < 0.05 as the screening criteria for significantly different metabolites. Before the annotation and analysis of KEGG pathways, the differential metabolites screened by positive and negative ion patterns were combined. The significant levels of metabolite enrichment in each pathway were analysed and calculated by Fisher's accurate test (Fisher's exact test) to determine the metabolic and signal transduction pathways that were significantly affected. To observe the expression of the differential metabolites annotated in the KEGG metabolic pathway, a KEGG metabolic pathway with more than 5 differential metabolites was selected to display the differential metabolites in the KEGG metabolic pathway in the form of a heat map [63, 64].

Abbreviations

AOX	alcohol oxidase
FLD	formaldehyde dehydrogenase
FGH	S-hydroxymethyl glutathione hydrolase
FDH	formate dehydrogenase
DPCs	DNA-protein crosslinks
Δfld	formaldehyde dehydrogenase-deficient strain
Δfgh	S-hydroxymethyl glutathione dehydrogenase-deficient strain
Δfdh	formate dehydrogenase deficient-strain
DEGs	differentially expressed genes
DAS	dihydroxyacetone synthase
GL	GS115 versus KO_FLD
GG	GS115 versus KO_FGH
GD	GS115 versus KO_FDH
ACAS	acetyl-coenzyme A synthetase
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
FBA	fructose-bisphosphate aldolase
CAT	catalase
GPX	glutathione peroxidase
GSR	glutathione reductase
COX7C	cytochrome c oxidase subunit 7c
COX10	haem o synthase
ROS	reactive oxygen species
GSH	glutathione
GSSG	oxidized glutathione
metE	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
CTH	cystathionine gamma-lyase
AOC	amine oxidase
amiE	amidase
PRODH	proline dehydrogenase
GCLC	glutamate-cysteine ligase catalytic subunit
GST	glutathione S-transferase
DUG	Cys-Gly metallodipeptidase
UPS	ubiquitin–proteasome system

Declarations

-Ethics approval and consent to participate

Not applicable.

-Consent for publication

Not applicable.

-Availability of data and materials

The RNA sequence datasets generated and/or analysed during the current study are available in the [NCBI Sequence Read Archive (SRA)] repository, [<https://www.ncbi.nlm.nih.gov/biosample/>]. Our recent submission to the BioSample database has been successfully processed and will be released on the date specified. BioSample accessions: SAMN20981851, SAMN20981852, SAMN20981853, SAMN20981854. Temporary Submission ID: SUB10230863. Release date: 2022-09-08, or with the release of linked data, whichever is first.

All metabolomics data generated or analysed during this study are included in this published article [and its supplementary information files].

-Competing interests

The authors declare that they have no competing interests.

-Funding

This report research was supported by the National Key R&D Program of China [2018YFA0901500]. It bears the cost of testing the transcriptome and metabolome and the labor cost of the researchers.

-Authors' contributions

YY carried out the whole experiment design, operation and data analysis, and was a major contributor in writing the manuscript. JY conducted experimental operations and data analysis of the transcriptome. FZ provided *Pichia pastoris* CRISPR/Cas9 technology and analyzed some transcriptome data. YL guided the conception and design of the experiment. SH guided the research throughout the whole process and analyzed and summarized all experimental contents. All authors read and approved the final manuscript.

-Acknowledgements

Not applicable.

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Methane
level of

Figure 2

Analysis of the differences between dissimilation pathway knockout and wild-type strains. A. The difference in biomass of 1 OD/mL fermentation broth in YPD and/or 1% YPM for 12 h. B. Number of DEGs between dissimilation pathway knockout and wild-type strains under methanol culture conditions. C. Venn diagrams of DEGs between dissimilation pathway knockout and wild-type strains under methanol culture conditions. D. Differential metabolite ratios of Δfld and Wild-type strain under methanol culture 24h conditions ($q \leq 0.05$).

Figure 3

Analysis of the difference of oxidative phosphorylation pathway under methanol culture conditions. A. Dialogue with oxidative phosphorylation in *Pichia pastoris*. B. DEGs of oxidative phosphorylation between dissimilation pathway knockout and wild-type strains.

Figure 4

Comparative transcriptome analysis of glutathione and amino acid metabolism under methanol culture conditions. The arrow indicates the comparative transcription level of the dissimilation pathway knockout and wild-type strains ($q \leq 0.05$).

Supplementary Files

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- [Additionalfile1.KEGGclusteringanalysisofdifferentialmetabolitesbetweenformaldehydedehydrogenaseknockoutstrainsversuswildstrains.pdf](#)
- [Additionalfile2DEGscusteringinaminoacidmetabolism.pdf](#)