Metabolic mechanism of *Saccharomyces cerevisiae* under different physiological conditions based on non-stationary ^{13}C metabolic flux analysis

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**Metabolic mechanism of *Saccharomyces cerevisiae* under different
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Abstract: Crabtree effect is well known for *Saccharomyces cerevisiae*, and is defined as glucose-induced repression of respiratory flux. Even though a number of hypotheses have been formulated, its triggering mechanisms are still unknown. At present, the information about intracellular metabolic flux can be obtained by the ^{13}C isotope labeling experiments. ^{13}C metabolic flux analysis(^{13}C -MFA) is a traditional method for calculating metabolic flux based on isotopic steady state. Another new method (INST- ^{13}C -MFA: Isotopically nonstationary metabolic flux analysis) based on isotope non-steady state is being used by researchers. In this review, we have chemostatized *S. cerevisiae* at three different dilution rates ($D=0.12, 0.22, 0.32 \text{ h}^{-1}$) and obtained the metabolic flux distribution of the intracellular central carbon metabolic of *S. cerevisiae* using INST- ^{13}C -MFA. Combined with the metabolome and metabolic fluxome data, we found obvious metabolic flux shift under the three different physiological states. In this process, pyruvate decarboxylase, ethanol dehydrogenase and acetyl-CoA synthase(AcCoA) catalyzed reactions were key points. Negative correlation between relative flux of embden meyerh of pathway(EMP) and tricarboxylic acid cycle(TCA) and biomass yield, while positive correlation for pentose phosphate pathway(PPP) were observed. Yield of acetate and glycerol did not change significantly, while that of ethanol increased sharply. In the central carbon metabolism (CCM), most of the carbon flux (70%) was

directed to the EMP. At the same time, the energy charge increased with dilution rate, and the cell's energy supply mode gradually shifted from oxidative respiration to substrate level phosphorylation mode.

Key words: *Saccharomyces cerevisiae*; INST-¹³C-MFA; Crabtree effect; central carbon metabolic; metabolic flux;

1. Introduction

The constantly decreasing amount of fossil resources, global warming as well as increasing public demand for sustainable production processes drive the research in the field of bio-based alternatives (Burk and Van Dien 2016). The use of microorganisms for different kinds of applications has a long tradition, starting with alcoholic fermentation as well as acetic acid production (Zhang, Sun, and Ma 2017; Zheng et al. 2018). The product scope has broadened tremendously over the past decades to various high-volume chemicals, materials, and fuels, and high-value molecules for human health and nutrition (Schwechheimer et al. 2016; Becker and Wittmann 2012; Becker et al. 2015; Becker and Wittmann 2016). For *Saccharomyces cerevisiae*, even under aerobic conditions, it is an industrial microorganism that still undergoes ethanol fermentation when the glucose concentration is high (De Deken 1966). Simultaneously, the yeast *Saccharomyces cerevisiae* is one of the major workhorses used in biotechnology for producing pharmaceuticals,

biofuels (Hashem and Darwish 2010), and bulk chemicals (Willke and Vorlop 2004). More importantly, *S. cerevisiae* is also an important model organism for studying the physiology (Pereira, Eleutherio, and Panek 2001), genetics and metabolic mechanisms of eukaryotes (Castrillo et al. 2007). As we all know, metabolism is among the most strongly conserved processes across all domains of life and is crucial for both bioengineering and disease research (Hackett et al. 2016). Estimating rates of reactions of metabolic network accurately is crucial for metabolic application (Suarez-Mendez et al. 2016). This is because that the knowledge of intracellular reaction rates (fluxes) is crucial to understand how cells utilize nutrients and how they adapt the fluxes in response to environmental or genetic perturbations (Lehnen, Ebert, and Blank 2017). Crabtree effect is well known for *Saccharomyces cerevisiae*, and is defined as glucose-induced repression of respiratory flux. Even though a number of hypotheses have been formulated, its triggering mechanisms are still unknown. So, Crabtree effect has caused widespread concern, and it is significance to revealing its mechanism. The investigation of intracellular reaction rates has become accessible to a wide range of scientists, rather than exclusively experts, and has been applied to expand detailed knowledge on cellular physiology (Blank, Lehmbeck, and Sauer 2005; Long and Antoniewicz 2014; Petersen et al. 2000), as well to guide metabolic engineering (Bartek et al. 2011; Stephanopoulos 1999; Toya

and Shimizu 2013) and biomedical research (Boghigian et al. 2010).

At present, the most efficient and accurate means of obtaining the data of intracellular metabolic flux is the ^{13}C isotope labeling experiments. ^{13}C -metabolic flux analysis (^{13}C -MFA) has emerged over the past 20 years as an important approach to assess in vivo metabolic phenotypes (Sauer 2006; Wiechert 2001). In ^{13}C -MFA, after feeding of a ^{13}C -labeled carbon source into a cell culture, amino acid or intermediates are extracted and subjected to mass spectrometric analysis. It relies on least-squares regression of isotope labeling measurements and extracellular exchange rates to reconstruct comprehensive flux maps that depict the flow of carbon throughout intracellular metabolism (Young 2014). Based on the labeled information obtained by the isotope labeling experiment (wet experiment), the optimal evaluation value of the metabolic flux is obtained by computer iterative calculation and continuous fitting. Therefore, we need some software platforms for calculating the metabolic flux. At present, in order to promote the wider application of ^{13}C -MFA in the construction of metabolic network models and the calculation of metabolic fluxes, several publicly available software packages have been developed, which have: 13CFLUX2 (Weitzel et al. 2013), Metran (Yoo et al. 2008), OpenFLUX (Quek et al. 2009), INCA (Young 2014), OpenMebius (Kajihata et al. 2014).

In our review, OpenMebius (Open source software for Metabolic

flux analysis) software platform was used to calculate the intracellular metabolic flux of *Saccharomyces cerevisiae* at different dilution rates. When the metabolic steady state is reached, OpenMebius has been developed to perform INST- ^{13}C -MFA (isotopically nonstationary ^{13}C metabolic flux analysis) and conventional ^{13}C -MFA (isotopically stationary ^{13}C metabolic flux analysis) using a user-defined metabolic model (Kajihata et al. 2014). In OpenMebius, A metabolic model M can be automatically generated from a metabolic pathway and carbon transition network. Under the metabolic network model M , combined with the labeling information of the ^{13}C isotope, the optimal metabolic flux distribution map was obtained by nonlinear fitting. In our experiments, INST- ^{13}C -MFA (see Fig 1) was used to calculate the metabolic flux of *S. cerevisiae* at three different dilution rates ($D = 0.12, 0.22, 0.32 \text{ h}^{-1}$). Combining the data of metabolites and metabolic flux, we try to explain the mechanism of Crabtree effect and found that *S. cerevisiae* undergoes metabolic migration of intracellular central carbon metabolic under three different physiological conditions. Moreover, the metabolic flux of the EMP pathway, the PP pathway, and the TCA cycle (when the glucose uptake rate is set to 1) has a certain relationship with the yield of the biomass.

Fig1: Metabolic steady state and isotopically stationary. The isotopic labeling experiment is performed under metabolic steady state (the yellow dotted line indicates that metabolic steady is achieved after 5 residence times). After feeding ^{13}C -labelde glucose, isotopic labeling enrichment changes in a time-dependent manner and then reaches a stationary condition. Whereas *S. cerevisiae* are sampled under isotopically stationary conditions in conventional ^{13}C -MFA, time courses of isotopic labeling enrichment during an isotopically transient state are used for INST- ^{13}C -MFA.

2. Material and methods

2.1. Strain and culture conditions

We used the *Saccharomyces cerevisiae* CEN PK 113-7D strain purchased from EUROSCAR, activated in YPD solid medium, and the activated strain was stored in -80°C glycerol tubes. A bioreactor (Model:

Multifors Bacteria) manufactured by INFORS was used to culture the yeast. Trace metal solution in Delft medium: 3 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 4.5 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 4.5 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.3 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4 g/L $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 1 g/L H_3BO_3 , 0.1 g/L KI, 19 g/L $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$; Vitamin solution in Delft medium: 0.05 g/L d-Biotin, 1 g/L D-Pantothenic acid hemicalcium salt, 1 g/L Thiamin-HCl, 1 g/L Pyridoxin-HCl, 1 g/L Nicotinic acid, 0.2 g/L 4-aminobenzoic acid, 25 g/L m-Inositol. The solution of *Saccharomyces cerevisiae* was taken from the glycerol tube and streaked on the plate of the YPD solid medium, placed in a 30 ° C incubator, and cultured for 48 hours; Pick a single colony of yeast from the plate and inoculate it in a tube containing 3 ml of Delft medium [7.5 g/L $(\text{NH}_4)_2\text{SO}_4$, 14.4 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g/L glucose, 1 ml/L vitamin solution, 2 ml/L trace metal solution], the tube was incubated for 12 hours in a shaker at a speed of 220 rpm and a temperature of 30 ° C. After that, transfer the fermentation broth in the test tube to a shake flask containing 100 ml of Delft medium that is consistent with the medium in the test tube, the shake flask was incubated for 12 hours in a shaker at a speed of 220 rpm and a temperature of 30 ° C. Finally, the fermentation broth in the shake flask is transferred to a bioreactor containing 600 ml of Delft medium [5 g/L $(\text{NH}_4)_2\text{SO}_4$, 3 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g/L glucose, 1 ml/L vitamin solution, 1 ml/L trace metal solution]. The

aeration was performed using pressurized air at 0.6 L/min (approx. 1vvm) and a stirrer speed of 700 rpm. The broth was maintained at PH 5.0 by adding 2M KOH, the temperature was controlled at 30 ° C and pressure was kept at 0.3 bar.

2.2. Chemostat cultivation

When the dissolved oxygen rises and the carbon-dioxide escape rate(CER) falls for the second time (the two occur at the same time), that indicates reactions reach the end of the batch fermentation stage due to the "secondary growth" phenomenon of *Saccharomyces cerevisiae*. After that it begins to transfer to the chemostat culture. The composition of Delft medium added into bioreactor is the same as that of the batch fermentation medium during the chemostat culture, besides the concentration of glucose is changed to 7.5 g/L. The rest of the fermentation parameters are consistent with the batch fermentation. Ethanol was not added to the medium since no oscillations were observed under these conditions. We performed chemostatization experiments at three different dilution rates ($D = 0.12, 0.22, 0.32 \text{ h}^{-1}$). After culturing five elution volumes, we considered that metabolic steady was reached (van Eunen et al. 2011). At this time, the parameters such as CER, oxygen escape rate (OUR), and dissolved oxygen are basically unchanged, these phenomena also prove the state of achieving metabolic homeostasis. At the same time, we began to switch the substrates to a medium containing

^{13}C -labeled glucose. Ingredients in the medium are consistent with the medium of chemostat, the only difference between these two media was that glucose labeled with ^{13}C and U- ^{13}C : $1\text{-}^{13}\text{C} = 4:1$. The feed rate and parameters of fermentation remain constant, ensuring that the *Saccharomyces cerevisiae* in the bioreactor is still stay the state of metabolic steady after switching substrates. ^{13}C -glucose is maintained for 1/10 elution volumes, we sampling from the broth rapidly to obtain data about the changes of isotopically labeled information over time during this process.

2.3. Rapid sampling

An in-house-made rapid sampling device was applied for the rapid sampling after switching the substrate. The Single Chip Microcomputer technique was employed to control the opening and closure of three valves. The device was optimized so that it takes less than 1 s to sample a volume of ~ 1 mL broth.

Considering that the turnover time of intracellular metabolites is very short(Sauer 2006), in order to accurately obtain the data of changes in the mass distribution vector (MDV) of intracellular metabolites over time, during the period of supplementation of ^{13}C -glucose, we conducted sampling densely in the early stage: first 40s, sampling every 5s. Taking samples every 20s and takes about 5 samples in the middle. The interval between the later sampling is longer: sampling every 1 minute, or

sampling every 5 minutes. The entire process of rapid sampling takes approximately 20 samples. At different dilution rates, the time of the rapid sampling process is also different. At the dilution rates $D=0.12, 0.22, 0.32 \text{ h}^{-1}$, the rapid sampling process lasts for 60, 30, and 20 minutes, respectively.

2.4. Data acquisition

The concentration of biomass was determined by a gravimetric method (dry weight). When metabolic steady state is reached, 10 ml of broth were filtered through a pre-dried and pre-weighted membrane ($0.8 \mu\text{m}$, 0.47 mm). The filter containing the biomass on the surface was placed in a microwave oven whose power is 700 W and dried again for 3 min. After cooling to room temperature, it was weighed. We have done pre-experiment using this method before, and the weight of the filter is basically unchanged after 2.5 min, it can be considered completely dry. The biomass concentration (C_x) can be used to calculate the glucose consumption rate (q_s) and the product rate of extracellular metabolites ($q_{\text{eth}}, q_{\text{gly}}, q_{\text{ace}}$) later.

2.4.1. Extracellular metabolites

In our experiments, the concentration of extracellular by-products was quantized, which determines the rate of exchange reactions and provides indispensable data for calculating metabolic fluxes. When reach the metabolic steady state, we believe that the concentrations of both

intracellular and extracellular metabolites are in a stable state. For quantification of extracellular metabolite concentration, about 1.5 ml of the broth was taken out from the bioreactor, placed in a pre-cooled (-20°C) syringe, filtered through a $0.22\ \mu\text{m}$ filter (Mashego et al. 2006), and the filtrate was used for analysis of extracellular by-products and residual glucose. Extracellular (filtrate) glucose, ethanol, acetate and glycerol concentrations were determined by one/all of the following two methods: HPLC, or enzymatic assays (Canelas et al. 2011).

The extracellular glucose concentration was measured by a glucose reagent kit (Huili biotech co., LTD, China). The glucoamylase concentration was determined by NPG method. $11.5\ \mu\text{L}$ sample was mixed with $131.5\ \mu\text{L}$ p-NPG substrate (pre-warmed for 5 min at 37°C , $2\ \text{g/L}$ 4-Nitrophenyl- α -d-glucopyranoside, Sigma N-1377). After incubation at 37°C for 20 min, the reaction was stopped by adding $57\ \mu\text{L}$ $0.3\ \text{M}$ Na_2CO_3 with immediate absorbance reading at 405 nm on a plate reader (MKIII plate reader, Thermo-Fisher, USA).

In this study, the extracellular metabolites detected are mainly ethanol, acetate and glycerol. The concentrations of extracellular metabolites were measured using HPLC, using an Agilent Technologies Hi-Plex H column ($300 \times 7.7\ \text{mm}$) equipped with a guard column ($50 \times 7.7\ \text{mm}$). The mobile phase used was $3\ \text{M}$ H_2SO_4 , with a flow rate of $0.4\ \text{L/min}$.

2.4.2. Intracellular metabolites

For quantification of intracellular metabolites concentrations, about 1 ml broth samples were rapidly withdrawn and quenched in pre-weighted tubes containing 20 ml cold (-40°C) pure methanol (Canelas et al. 2009) followed immediately by vigorous vortexing. The quenched samples were rapidly weighed and poured into a filtration device containing a cellulose membrane ($0.8\ \mu\text{m}$, $0.47\ \text{mm}$). Subsequently, a vacuum was applied followed by an immediate additional washing step with 10 ml cold methanol (-40°C). The filter containing the cold washed biomass was then transferred into a 50 ml-centrifuge tube containing 20 ml of preheated (75°C) aqueous ethanol solution (75% v/v). 100 μL of ^{13}C cell extract was added to the tube as an internal standard (Wu et al. 2005). The tube containing the sample was then tightly closed, shaken vigorously, and placed into a water bath at 95°C during 3 min for intracellular metabolite extraction. After, the tubes were then cooled using ice bath and the filter was removed. This extract was then concentrated by complete evaporation of ethanol-water mixture under vacuum (Mashego et al. 2004). Finally, 600 μl of milliQ water was added to the centrifuge tube, and after thoroughly mixing and dissolved, the solution was transferred to a 1.5 ml EP tube placed into a screw-capped polypropylene vial and stored at -80°C until further analysis. Samples were analyzed by LC -MS/MS (van Dam, Ras, and ten

Pierick 2011; Sollner et al. 2009) and/or GC-MS (de Jonge et al. 2011; Cipollina et al. 2009).

2.4.3. Calculation methods

The biomass specific uptake and secretion rates were reconciled using the approach of Verheijen (2010). A constant elemental biomass composition and molar weight of 26.4 g_{DCW}-C-mol⁻¹ was used for all conditions.

The biomass specific uptake and secretion rates (q : mmol/g_{DCW}/h), were calculated from the respective dynamic mass balance using polynomial fits for the measured biomass and extracellular metabolite concentrations as a function of time (i = biomass, ethanol, acetate and glycerol):

$$\frac{dC_i(t)V_L}{dt} = F_{L,in}C_{i,in} - F_{L,out}C_i(t) + q_i(t)C_x(t)V_L$$

Here, C_i is concentration of compound i in the reactor, V_L is broth volume, $F_{L,in}$ is medium feed flow, $F_{L,out}$ is effluent flow, q_i is biomass specific consumption/secretion rates for compound i , C_x is biomass concentration.

The biomass specific O₂ uptake and CO₂ evolution rates were calculated from the pseudo steady state gas phase balances (j = O₂, CO₂) (where $F_{g,out}$ was obtained from the $F_{g,in}$ and the N₂ balance):

$$0 = F_{g,in}Y_{j,in} - F_{g,out}Y_{j,out} + q_j(t)C_x(t)V_L$$

Where C_x is biomass concentration, V_L is broth volume, $F_{g,in}$ is gas

flow in, $F_{g,out}$ is gas flow out, $y_{j,in}$ is concentration of compound j in gas flow in, $y_{j,out}$ is concentration of compound j in gas flow out, q_j is specific consumption/secretion rates for compound j.

2.4.4. Mass isotopomer measurements

Samples for quantification of mass isotopomer enrichments were taken following the same procedure described above for concentration of extra- and intracellular metabolites with the exception that no ^{13}C cell extract was added. The mass isotopomer samples were then analyzed by LC-MS/MS (van Winden et al. 2005).

2.4.5. Estimate metabolic flux

Under the Linux operating system, OpenMebius is run using MATLAB (MathWork, Natick, MA, USA), which builds a metabolic model (see Supplementary material 1) based on metabolic network model and carbon atom migration model. Under this metabolic model, we need to provide data about the concentration of metabolites (all metabolites contained in the model) and the rate of exchange reactions. At different dilution rates, the rate of intermediates converted into biomass/amino acids in the exchange reaction is calculated based on the equation of bacterial synthesis. In this way, we can obtain the simulated value of a series of the mass distribution vector (MDV_{sim}) of intracellular metabolites (Antoniewicz, Kelleher, and Stephanopoulos 2007; Kajihata et al. 2014). Finally, the MDV_{sim} is compared with the experimental value

MDV_{exp} obtained by the labeling experiment to find the group which shows the optimal fitting effect, and that is the data of the metabolic flux we need. We made a simple summary explained the entire process of calculating metabolic flux, the steps of the method are shown in below (see Fig 2).

Fig 2: Schematic diagram of the calculation of metabolic flux by the INST-MFA method.

Currently available software tools, namely OpenMebius, are used for model specification, flux estimation, and statistical analysis. Initial guesses of fluxes and pool sizes are needed for the first iteration (iter) only. The flux map generated is a snapshot of metabolism during the course of the isotope labeling experiments (ILEs).

3. Results

3.1. Carbon-balance check

At three different dilution rates, we performed extracellular sampling and measured the concentration of extracellular metabolites when

metabolic steady state was reached. The data are shown in Table 1. It can be seen that a small portion of acetate and glycerol are secreted from the yeast at different dilution rates, and the concentration of them does not change significantly with changes in specific growth rate. For this, we calculated the C-balance at these three dilution rates. When $D=0.12, 0.22, 0.32 \text{ h}^{-1}$, the C-balance is 104.23%, 88.16%, and 108.09%, respectively. Among them, the C-balance at a dilution rate of 0.22 h^{-1} is not strictly observed

Table 2 shows the specific formation/consumption rates (unit: $\mu\text{mol gDCW}^{-1} \text{ h}^{-1}$) for each metabolites at different dilution rates. It can be seen that the specific consumption rate of glucose increases with the increase of specific growth rate. When D increases from 0.12 h^{-1} to 0.32 h^{-1} , the specific consumption rate of glucose increases by nearly 3 times.

Table 1

Macroscopic measurements for different dilution rate.

Dilution rate (h^{-1})	0.12	0.22	0.32
Biomass concentration (gdcwL^{-1})	3.42 ± 0.01	3.66 ± 0.01	2.44 ± 0.01
Residual Glucose concentration (mM)	0.36 ± 0.03	0.38 ± 0.03	1.37 ± 0.05
Ethanol concentration (mM)	b.d.	b.d.	29.73 ± 0.20
Acetate concentration (mM)	2.72 ± 0.15	1.35 ± 0.09	3.12 ± 0.15
Glycerol concentration (mM)	1.35 ± 0.17	0.55 ± 0.05	1.24 ± 0.10

b.d: below detection limit.

Table 2

The specific formation/consumption rates (unit: $\mu\text{mol g}_{\text{DCW}}^{-1} \text{ h}^{-1}$) for each metabolites.

Dilution rate (h^{-1})	0.12	0.22	0.32
Biomass	4550 ± 10	8134 ± 15	11469 ± 17
Glucose ($-q_s$)	1460 ± 15	2429 ± 25	5049 ± 45
Ethanol (q_{eth})	0	0	3752 ± 103
Acetate (q_{ace})	97 ± 2	80 ± 4	393 ± 5
Glycerol (q_{gly})	48 ± 1	33 ± 2	157 ± 2
CO_2 (q_c)	4242 ± 103	4457 ± 146	12988 ± 208

3.2. Analysis of assimilated carbon distribution

At three different dilution rates, we did a simple analysis for the distributions of carbon assimilated by *Saccharomyces cerevisiae*. Based on absolute quantification of intracellular metabolites, divided into five metabolite groups (AAs, EMP, PPP, TCA, and Energy factors) and calculated the proportion of carbon content in each metabolite group. We can see that the carbon content of amino acids is much higher than other metabolite groups at three dilution rates from the figure 3, which indicating that amino acids in yeast are the largest metabolic pool for storing carbon sources. The carbon content of the two metabolite groups (EMP and PPP) shows a certain increase with increasing in the specific

growth rate. We analyzed the reason for this phenomenon is that, the rate for synthesis of bacteria is faster at high specific growth rate, and more intermediates (G6P, R5P, NADPH, etc.) produced by EMP and PPP are needed. At the same time, we see a significant increase in the carbon content of the TCA cycle when $D = 0.12 \text{ h}^{-1}$. Combined with the distributions of metabolic flux below, we know that the reason is most of the pyruvate from cytoplasm is converted to acetyl-CoA at this dilution rate, which then enters the mitochondria to participate in the TCA cycle, hence resulting in that the carbon content of TCA when $D = 0.12 \text{ h}^{-1}$ higher than other dilution rates.

Fig 3: Distribution of carbon content at different dilution rates. Relative carbon content grouped by metabolite class with respect to the carbon consumed by *S. cerevisiae* at different growth rates.

The abscissa represents five different metabolite groups. The ordinate indicates the carbon

content.

3.3. Free amino acid

From the above analysis, the intracellular free amino acid is the largest metabolic pool for storing carbon sources in yeast, and the intracellular free amino acids are converted from intermediates produced in the central carbon metabolic pathway (e.g., alanine – pyruvate, glutamate – α -ketoglutarate, and aspartate – oxaloacetate). The analysis for free amino acids may provide a novel direction for our study of metabolic metabolism, which may help us to further understand the regulation of microbial metabolism. Therefore, it is particularly important to analyze the trend of changes in the concentrations of intracellular free amino acids at different dilution rates.

As we can see from the following two figures (Fig 4 and Fig 5), the concentrations of glutamic acid, lysine, glutamine, proline, serine, aspartic acid and asparagine increase with increasing specific growth rate, where glutamine, glutamic acid and aspartic acid has increased dramatically. The concentration of tyrosine and phenylalanine decreased with increasing specific growth rate, and the concentration of phenylalanine was significantly changed when the dilution rate from 0.12 to 0.22 h⁻¹. In this regard, we can estimate that the precursors formed free amino acids whose concentrations show obviously changes have

undergone significant changes under different physiological conditions. It may be related to the enzymes at protein level and the genes at transcription level, which have undergone corresponding changes, and that provides a research direction for our subsequent analysis about metabolic regulation of yeast. At the same time, we found an interesting phenomenon that the change of glutamine and glutamic are nonlinear while the change of aspartate is linear obviously.

Fig 4 and Fig 5: Changes in some intracellular free amino acids at different dilution rates. The abscissa indicates the specific growth rate (h^{-1}). The ordinate indicates the concentration ($\mu\text{mol gpcw}^{-1}$).

3.4. Extracellular glucose

In the bioreactor, the enrichment of extracellular ^{13}C -glucose is changes over time after the substrate is switched to ^{13}C labeled glucose. The carbon enrichment of extracellular glucose in time is influenced by: the ^{13}C feed flow rate, the specific glucose uptake rate, and the residual ^{12}C -glucose concentration. About this, we made a simple analysis for the process that how the carbon enrichment of extracellular glucose is changing. We calculated the corresponding turnover time (τ) at three dilution rates $D = 0.12, 0.22, 0.32 \text{ h}^{-1}$, which is 257, 155, and 400 sec, respectively. Accordingly, the fastest extracellular glucose enrichment was observed at $D=0.22 \text{ h}^{-1}$ and that is consistent with the findings of others (Suarez-Mendez et al. 2016), while the slowest was at $D=0.32 \text{ h}^{-1}$. For the reason why the slowest extracellular ^{13}C glucose enrichment was at $D = 0.32 \text{ h}^{-1}$, we believe the main reason is that the extracellular residual glucose concentration is too high at this dilution rate. In addition, we also studied the relationship between extracellular ^{13}C glucose enrichment and τ , it shown in the figure 7. We found that the trend of extracellular ^{13}C glucose enrichment and τ was basically the same for three different specific growth rates. After 5τ , all the carbon enrichment reached 80%, and it also approached stability.

Fig 7: Carbon enrichment of extracellular glucose. Dynamics of ^{13}C enrichment at different dilution rates as function of time normalized by the turnover time of extracellular glucose pool (experimental time/ τ). The abscissa represents time and the time was normalized to τ . The ordinate indicates average enrichment of extracellular ^{13}C -glucose.

3.5. Carbon flux distribution at the G6P branch

Flux maps at three different dilution rates are shown in supplementary material 2. We analyze the distribution of the metabolic flux on the G6P node (see Table 3). Most of the carbon influx at the G6P node was directed to the EMP pathway for these three different dilution rates. Especially at a high specific growth rate ($D=0.32 \text{ h}^{-1}$), and it reached the maximum value which is 77.31% (the glucose uptake rate is set to 1). That is consistent with the results of previous experiments (Suarez-Mendez et al. 2016), and it turns out that the excess carbons were generated pyruvate via the EMP pathway, and then converted to ethanol

subsequently. Therefore, ethanol can be detected extracellularly at a high specific growth rate. At the same time, we found that the flux ratio of the PP pathway fluctuated between 10.44% and 14.68% with no significant changes. We compared the proportion of flux on PP pathway with other's works. The similarity is that the PP pathway ratio is 14.7% when $D=0.2 \text{ h}^{-1}$, and the difference is that their value is 3.8% while our value is 14.15% when $D=0.1 \text{ h}^{-1}$ (Suarez-Mendez et al. 2016). We suspect that there may be differences in the species, culture system, or labeling substrate.

Table 3

Carbon flux distribution at the G6P node (all values are relative and glucose uptake rate set to 1).

Dilution rate (h^{-1})	0.12	0.22	0.32
PPP split ratio	0.1415	0.1468	0.1044
EMP split ratio	0.7305	0.6786	0.7731
Biomass split ratio	0.1280	0.1747	0.1243

3.6. TCA cycle

We list the flux proportions of the TCA cycle at three different physiological states (see figure 8). It can be found that the flux ratio of TCA cycle gradually decreases as the dilution rate increases, and the trends of decrease are significant. Therefore, it can be judged that the yeast undergoes metabolic migration due to its own regulation during the transitions of dilution rate, and some carbon do not enter the

mitochondria to participate in the TCA cycle. From figure we can see that most of the carbon flux were involved in the TCA cycle when $D = 0.12 \text{ h}^{-1}$ at these three dilution rates. However, it was the opposite when $D = 0.32 \text{ h}^{-1}$.

Fig 8: Carbon flux distribution at TCA cycle and all values are relative and glucose uptake rate set to 1.

3.7. The yield of biomass and by-product

In our research, we found an interesting phenomenon as we put the yield of biomass and the relative flux of EMP/PP pathway at these three dilution rates on one graph (see Figure 9). And we will find that the yield of biomass increased and then decreased with the dilution rate increasing. The reason why the biomass yield was decrease is that more carbons are converted to ethanol, not biomass at higher dilution rates. At the same time, we also found that the change of the relative flux on PP pathway is

consistent with the change of the yield of biomass. However, it is the opposite for EMP, and the result about that is worthy of further research and analysis. The PP pathway mainly provides essential precursors for the synthesis of biomass (e.g., R5P, X5P, NADPH, etc.), therefore, the yield of biomass is positively correlated with the relative flux of PP pathway. And the EMP provides the precursor substance pyruvate, were converted into extracellular by-product (e.g., ethanol, acetate). So, the yield of biomass is inversely correlated with the EMP relative flux.

After analyzing the biomass yield, then we analyzed the yield of extracellular by-products (see Figure 10). From the figure we can see that the yield of extracellular products acetic acid and glycerol did not change significantly at these three dilution rates. Therefore, it can be inferred that the metabolic flux (relative flux) of producing acetate and glycerol did not change significantly during the changes on dilution rate. There is no ethanol production at the first two low dilution rates, but the ethanol yield rises sharply at high dilution rates ($D = 0.32 \text{ h}^{-1}$). And this provides a research direction for the "metabolic migration" we mentioned earlier. Combining the yield of biomass and the yield of by-products, we found that a large amount of carbons enter the EMP pathway which further converted into ethanol, then resulting in the declines of biomass yield and PPP flux (relative flux) under the high specific growth rate ($D=0.32 \text{ h}^{-1}$).

Fig 9: The relationship between the yield of biomass and the relative flux of EMP/PPP pathway at these three dilution rates. The abscissa indicates the dilution rate (h^{-1}), the main ordinate indicates the biomass yield ($\text{C-mol C-mol-glucose}^{-1}$), secondary ordinate indicates the relative flux of EMP/PPP pathway (when glucose uptake rate set to 1). Fig 10: The relationship between the yield of by-products and dilution rates. The abscissa indicates the dilution rate (h^{-1}), the ordinate indicates the yield of by-products ($\text{C-mol C-mol-glucose}^{-1}$).

3.8. ATP balance and NADPH balance

In addition, we also analyzed the balance of ATP (see Fig 11) and NADPH (see Fig 12). For the generation of ATP, it is mainly derived from substrate level phosphorylation and electron transport chain, and the electron transport chain is based on $P/O=1$ (Rigoulet et al. 1989). The consumption of ATP is mainly used to maintain the growth of the yeast, and it was estimated by using a constant of 69.2 mmol ATP/g_{DCW} (Forster

et al. 2003). It can be seen that most of the ATP is produced by reactions on the electron transport chain at different dilution rates. And the ratio of ATP produced by substrate-level phosphorylation are 17.08%, 22.23%, 32.43% when $D=0.12$, 0.22, 0.32 h^{-1} respectively, it is obvious that the ratio of ATP produced by substrate-level phosphorylation increase gradually. Meantime, the rate of ATP production increases gradually as the dilution rate increases. The reason is that more carbons enter the yeast to participate in the reaction to provide ATP with the glucose consumption rate increases. ATP balance was closed strictly at $D=0.22$ and 0.32 h^{-1} , but the balance was not closed at $D=0.12\text{h}^{-1}$.

Fig 11: Production and consumption of ATP. The production and consumption rates ($\text{mmol g}_{\text{DCW}}^{-1} \text{h}^{-1}$) of ATP were estimated using the metabolic flux distribution under three dilution rates. (A). $D=0.12 \text{ h}^{-1}$ (B). $D=0.22 \text{ h}^{-1}$ (C). $D=0.32 \text{ h}^{-1}$

The production of NADPH mainly comes from the reactions on the PPP pathway. And the consumption of NADPH is mainly used for the synthesis of biomass, such as synthetic nucleotides, amino acids, lipids, etc. We used a constant of $9.31 \text{ mmol NADPH/g}_{\text{DCW}}$ to characterize the consumption of NADPH (Bruinenberg et al. 1986). The rate of NADPH

formation increases with increasing dilution rates due to an increase in flux (absolute flux) over the PPP pathway. However, at three dilution rates, the balance of the NADPH production and consumption rates, estimated from metabolic flux distribution, was not closed. It has been explained that this is because some of the NADPH is transported into the mitochondria and then participate in the reactions on the electron transport chain to generate ATP (Sollner et al. 2009).

Fig 12: Production and consumption of NADPH. The production and consumption rates (mmol g_{DCW}⁻¹ h⁻¹) of NADPH were estimated using the metabolic flux distribution under three dilution rates. (A). D=0.12 h⁻¹ (B). D=0.22 h⁻¹ (C). D=0.32 h⁻¹

4. Discussion

It shows that there is a significant change of the flux distribution of intracellular central carbon metabolic under different physiological conditions by using the ¹³C metabolic flux analysis of *Saccharomyces cerevisiae*. And the flux distributions were closely related to the environment surrounding bacteria. In our research, we think it was accompanied with metabolic migration when *S. cerevisiae* suffer from low specific growth rate to high specific growth rate. Our results indicate there was a sharp rise in ethanol yield and a sharp drop in TCA flux (relative flux), it is consistent with results of previous research (Frick and Wittmann 2005). Moreover, we also found that the bacterial yield has a certain relationship with the metabolic flux (relative value) on the EMP and PP pathways.

- i. Our works show that the changes in physiological status have a significant impact on the flux distribution of EMP, PPP, and TCA (see supplementary material 2). The trends of flux on the PPP against with the EMP, the two pathways have a certain relationship with biomass yield. As analyzed above, the biomass

yield was negatively correlated with the flux (relative flux) of the EMP and positively correlated with the flux (relative flux) of the PPP, and that was not reported previously. We think it is related to the functions of various metabolic pathways in yeast. We guess the reason is that PPP mainly provides essential precursors for the synthesis of biomass while the EMP provides the unique precursor substance pyruvate converted into extracellular by-product (e.g., ethanol, acetate) during the process. For the TCA cycle, the metabolic flux (relative flux) shows a significantly decreasing trend as the dilution rate increases. At the same time, we observed that the Crabtree effect occurred during the process of increasing dilution rate. It is the yield of ethanol has increased dramatically, which provides a direction for us to study the metabolic migration. For the G6P node, the previous study about the enzyme activity of *Saccharomyces cerevisiae* under chemostat culture conditions showed that the activity of G6P dehydrogenase was slightly increased with $D=0.1 \text{ h}^{-1}$ increases to 0.4 h^{-1} (Serra, Strehaiano, and Taillandier 2003). In our work, at three dilution rates ($D = 0.12, 0.22, 0.32 \text{ h}^{-1}$), the rates of reaction catalyzed by G6P dehydrogenase was 0.0574, 0.0955, 0.1464 $\mu\text{mol/g DCW/sec}$, respectively. This is consistent with that reported by Seera (2003). G6P

dehydrogenase is the first and key enzyme in PP pathway. We found that the flux (relative value) of PPP calculated at a low specific growth rate by others is only a little higher than the high specific growth rate (Gombert et al. 2001), which is close to our results. At the same time, there are cases that the flux is much higher (Frick and Wittmann 2005). We suspect that the reason for this phenomenon maybe the differences in culture systems or strains (other's strain is *Penicillium chrysogenum*).

- ii. In *Saccharomyces cerevisiae*, pyruvate decarboxylase is a key enzyme that catalyzes certain reactions produced precursor substances for the TCA cycle. Pyruvate is catalyzed by pyruvate decarboxylase to form acetaldehyde which is catalyzed by acetaldehyde dehydrogenase to form acetate. And pyruvate catalyzed by acetyl-CoA synthetase to form acetyl-CoA which enters the mitochondria and participates in the TCA cycle. We found that the flux distribution around the pyruvate node has changed significantly under these three different physiological states. Especially, a large amount of pyruvate is converted to acetaldehyde and then participates in subsequent reactions at high dilution rate. This indicates that a large amount of pyruvate is still involved in the fermentation reaction when *S. cerevisiae* is in the state of producing ethanol, and it refuting the view that

only a small proportion of pyruvate is involved (Lei, Rotboll, and Jorgensen 2001). Rather, it supports the view that the flux distribution around the pyruvate node is related to “Crabtree effect” of *Saccharomyces cerevisiae* (Postma et al. 1989). The flux flowed into acetyl-CoA from pyruvate has been high, whether at low specific growth rates or at high specific growth rates. And it indicates that the activity of pyruvate decarboxylase has been in an excellent state all the time. Our work also shows that the concentration of pyruvate increases with increasing dilution rate, which is consistent with the previously reported results (Postma et al. 1989). We suspect that the reason may be that some pyruvate in the mitochondria is transferred to the cytoplasm for the synthesis of alanine.

- iii. By combining the relative values (glucose uptake rate set to 1) of the metabolic flux distribution acquired from OpenMebius and the rate of glucose consumption, we can obtain the absolute rate of the reaction in the central carbon metabolic of *Saccharomyces cerevisiae* at different growth rate. From the perspective of the absolute value of metabolic flux, we can further understand the relationship between some complex reactions in central carbon metabolic. And the specific consumption rate of glucose increases with the increase of specific growth rate, increases by

nearly 4 times (the specific consumption rate of glucose is 0.4056, 0.6748, 1.4026 $\mu\text{mol g}_{\text{DCW}}^{-1} \text{sec}^{-1}$ under $D=0.12, 0.22, 0.32\text{h}^{-1}$, respectively). Therefore, how the increased carbon sources are distributed in the central carbon metabolic is worth analysis. At these three dilution rates, the metabolic flux (absolute value) of the reaction catalyzed by pyruvate decarboxylase, acetaldehyde dehydrogenase, and acetyl-CoA synthetase is shown below (see Fig 13). As we can see from the figure, the metabolic flux associated with pyruvate decarboxylase increases sharply with increasing specific growth rate, while the metabolic flux associated with acetaldehyde dehydrogenase and acetyl-CoA synthetase keep steady nearly. Further, there was no significant change in the metabolic flux associated with acetyl-CoA synthetase at different dilution rates, and it indicates that the acetyl-CoA synthetase has reached a state of work saturation regardless of the low dilution rate or the high dilution rate. Therefore, the accumulated acetate is discharged from the cells, so that we can detect the by-product acetate extracellularly. Moreover, the rate of acetate production is gradually increased, and the specific production rate of acetate is 0.0271, 0.0450, 0.1093 $\mu\text{mol g}_{\text{DCW}}^{-1} \text{h}^{-1}$ at $D=0.12, 0.22, 0.32 \text{h}^{-1}$, respectively, which is closely related to that acetyl-CoA

synthetase has reached a state of work saturation. Aldehyde dehydrogenase and acetyl-CoA synthetase are the key enzymes for the synthesis of acetyl-CoA, which can further synthesize the amino acids and lipids required for the growth of the yeast. Studies have shown that the activity of acetaldehyde dehydrogenase and acetyl-CoA synthetase decreases in the in-vitro enzyme activity experiment when the metabolic state changes from respiration to fermentation (Serra, Strehaiano, and Taillandier 2003), this is also consistent with our results. These enzymes catalyze certain key reactions and have great significance for guiding the transformation of strains.

Fig 13: The metabolic flux (absolute value) of the reaction catalyzed by pyruvate decarboxylase, acetaldehyde dehydrogenase, ethanol dehydrogenase and acetyl-CoA synthetase. The abscissa represents

three different enzymes. The ordinate indicates the reaction flux (absolute value) ($\mu\text{mol gDCW}^{-1} \text{ h}^{-1}$)

catalyzed by the corresponding enzyme.

5. Conclusions

Crabtree effect is well known for *Saccharomyces cerevisiae*, and we also encountered this imagination in our work. However, its triggering mechanisms are still unknown. In general, the changes of environmental influences the metabolism-related regulatory factors which further affects the metabolic distributions of the entire central carbon metabolic in *S. cerevisiae*. When the physiological state changes from a low specific growth rate to a high specific growth rate, the metabolic regulation of *Saccharomyces cerevisiae* will change accordingly, such as enzyme activity, stress resistance or self-morphology, etc. These corresponding changes are controlled by complex transcriptional or post-transcriptional systems (Daran-Lapujade et al. 2004). One of the most important factors affecting the metabolic mechanism of *Saccharomyces cerevisiae* in different environments is the concentration of glucose in the medium (Rolland, Winderickx, and Thevelein 2002). It has been proven in our work, the concentration of glucose in the medium increased with increasing specific growth rate (when $D=0.12, 0.22, 0.32 \text{ h}^{-1}$, glucose concentration was 0.3567, 0.3839, 1.3706 mmol/L). Especially at $D=0.32 \text{ h}^{-1}$, the glucose concentration increased nearly 4 times. At the same time,

the distribution of intracellular metabolic flux has also changed a lot. It has been previously reported that the main signal triggering changes in intracellular metabolic flux may be the glucose suppression system (Frick and Wittmann 2005). As the specific growth rate increases, the metabolic flux (relative value) of the TCA cycle is reduced. It may be that the glucose suppression system affects some genes in the TCA cycle at the transcriptional level (Schuller 2003). Similarly, the specific rate of glucose consumption and the metabolic flux of EMP pathways increase significantly, and may also affect related genes at the transcriptional level (Carlson 1999). Recently, studies on metabolic distributions and transcriptional level regulation have shown that the intracellular mechanism of *Saccharomyces cerevisiae* may also be regulated by post-transcriptional levels (Daran-Lapujade et al. 2004). Through the above analysis, it can be known that the metabolic flux characterizes the results combined with the metabolic regulation of microorganisms at different levels. Therefore, metabolic flux is a quantitative description for the intracellular metabolic state. In our review, we cannot use the single factor such as metabolic flux distribution to analyze or explain the changes of the metabolic regulation mechanism of *Saccharomyces cerevisiae* under different environments. However, the absolute quantification of the metabolic distribution of *S. cerevisiae* in different environments helps us understand its regulatory mechanisms at the level

of metabolic flux. If combined with metabolic flux, proteome, transcriptome and other omics, it will help us to have a global understanding of the regulation of microbial metabolism.

Abbreviations: CDW, cell dry weight; ^{13}C -MFA: ^{13}C metabolic flux analysis; INST- ^{13}C -MFA, Isotopically nonstationary metabolic flux analysis; CCM, central carbon metabolism; AcCoA, acetyl-CoA synthase; EMP, embden meyerh of pathway; TCA, tricarboxylic acid cycle; PPP, pentose phosphate pathway; AMP, adenosine-monophosphate; ADP, adenosine-diphosphate; ATP, adenosine-triphosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; 6PG, 6-phosphogluconate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; Iso-CIT, isocitrate; AKG, α -ketoglutarate; SUC, succinate; FUM, fumarate; MAL, L-malate; NADPH, Nicotinamide Adenine Dinucleotide Phosphate.

Declarations

- Ethics approval and consent to participate

Not applicable

- Consent for publication

Not applicable

- Availability of data and materials

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

- Competing interests

The authors declare that they have no competing interests.

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- Authors' contributions

HL analyzed and interpreted the change of the ATP paradox and the NAD⁺/ NADH ratio. WS and PL designed and implemented U-13C labeled cell extracts. HL and MC extracted the intracellular metabolites. HL and JYX were major contributors in writing the manuscript. All authors read and approved the final manuscript.

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