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^{13}C-metabolic flux analysis of lipid accumulation in the oleaginous fungus *Mucor circinelloides*

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HIGHLIGHTS

- First report showing ^{13}C-metabolic flux analysis in *Mucor circinelloides*.
- Malic enzyme and ATP: citrate lyase played an important role in lipid accumulation.
- High lipid-producing strain showed higher flux in PPP and lower flux in TCA cycle.

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ABSTRACT

The oleaginous fungus *Mucor circinelloides* is of industrial interest because it can produce high levels of polyunsaturated fatty acid γ-linolenic acid. *M. circinelloides* CBS 277.49 is able to accumulate less than 15% of cell dry weight as lipids, while *M. circinelloides* WJ11 can accumulate lipid up to 36%. In order to better understand the mechanisms behind the differential lipid accumulation in these two strains, tracer experiments with ^{13}C-glucose were performed with the growth of *M. circinelloides* and subsequent gas chromatography–mass spectrometric detection of ^{13}C-patterns in proteinogenic amino acids was carried out to identify the metabolic network topology and estimate intracellular fluxes. Our results showed that the high oleaginous strain WJ11 had higher flux of pentose phosphate pathway and malic enzyme, lower flux in tricarboxylic acid cycle, higher flux in glyoxylate cycle and ATP: citrate lyase, together, it might provide more NADPH and substrate acetyl-CoA for fatty acid synthesis.

1. Introduction

During these years, much attention has been paid to the development of microbial oils, which could synthesize polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and γ-linolenic acid (GLA), and microbial oils has also being considered as potential sources of biofuels (Li et al., 2008). Oleaginous filamentous fungi *Mortierella alpina* and *Mucor circinelloides* have been used to produce AA and GLA respectively (Li et al., 2015; Tang et al., 2014), oleaginous microalgaes *Aurantiochytrium limacinum* and *Schizochytrium* sp. could produce high level of DHA (Li et al., 2015; Song et al., 2015). DHA and EPA are considered to be essential for the proper visual and neurological development of infants (Das and Fams, 2003; Ramakrishnan et al., 2010). They are also known to be positively associated with health throughout life, particularly by reducing the incidence of cardiovascular diseases in adults (Demaison and Moreau, 2002). GLA has beneficial effects for prevention or treatment of many diseases including inflammatory disorders, diabetes, cardiovascular disorders, cancers, and some other diseases (Ratledge, 2005). Hence, microbial oils, which are rich in PUFAs have a huge market with big potential and wide prospect in the future.

The filamentous fungus *M. circinelloides* is of industrial interest because it can produce high levels of polyunsaturated fatty acid

Abbreviations: G6P, glucose-6-phosphate; 6PG, 6-phospho-gluconate; Ru5P, ribulose 5-phosphate; F6P, fructose-6-phosphate; E4P, erythrose-4-phosphate; S7P, seduheptulose-7-phosphate; T3P, triose-3-phosphate; 3PG, 3-phoshoglycetate; PEP, phosphoenolpyruvate; PYR, pyruvate; AcCoA, acetyl-CoA; OAA, oxaloacetate; CIT, citrate; ICT, isocitrate; AKG, α-ketoglutarate; SUC, succinate; FUM, fumarate; MAL, malate; GOX, glyoxylic.

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GLA, which is the first commercial producer of microbial PUFA (Ratledge, 2004). The oleaginous fungus *M. circinelloides* has emerged as one of the model of oleaginous fungus because of the availability of genome sequence (http://genome.jgi.doe.gov/Mucci2/Mucci2.home.html) and well-developed tools for genetic engineering (Torres-Martínez et al., 2012). Although much work has been done to study the mechanism of lipid accumulation in oleaginous fungi (Ratledge and Wynn, 2002), a systems-level analysis and understanding of the mechanisms underlying lipid accumulation in oleaginous filamentous fungus *M. circinelloides* is still lacking. Our previous work demonstrated that *M. circinelloides* WJ11 can produce lipid up to 36% of cell dry weight (CDW), which is about two-fold of *M. circinelloides* CBS 277.49 (no more than 15% of CDW), and the activities of the biochemical pathways of these two strains have been compared (Tang et al., 2015). Although this in vitro biochemical activity analysis has revealed some insights about the differential lipid accumulation in these two fungi, the exact in vivo activities of pathways related to lipid accumulation in these two fungi is still not clear.

On the basis of ^{13}C-labeling experiments, metabolic flux analysis (MFA) as an integrated experimental and computational method emerged to identify the biochemical network of active reactions and to provide quantitative insight into the in vivo distribution of molecular fluxes throughout central carbon metabolism (Zamboni et al., 2009). The molecular mechanism of lipid accumulation in oleaginous microalga *Chlorella protothecoides* (Xiong et al., 2010; Wu et al., 2015), oleaginous yeast *Yarrowia lipolytica* (Wasylenko et al., 2015) and *Trichosporon cutaneum* (Liu et al., 2013b), as well as bacteria *Escherichia coli* (He et al., 2014) and plant material of maize embryos (Alonso et al., 2010) have all been studied with this technique. In order to better understand the mechanisms of lipid accumulation in oleaginous fungus *M. circinelloides*, therefore, in this study, the intracellular metabolic fluxes of high lipid accumulation strain *M. circinelloides* WJ11 was investigated using ^{13}C-labeled metabolic flux analysis. This study showed for the first time, the application of stable isotope-based intracellular flux quantification to estimate and understand the mechanisms involved in lipid metabolism in oleaginous filamentous fungus.

2. Methods

2.1. Strain and growth conditions

M. circinelloides WJ11 and CBS 277.49 were used in this study, strain *M. circinelloides* WJ11 was previously isolated in our laboratory from soil at Jiangnan University, and preserved in China Center for Type Culture Collection (CCTCC No. M 2014424) (Tang et al., 2015), strain *M. circinelloides* CBS 277.49 was purchased from Centraalbureau voor Schimmelcultures (CBS).

K & R medium (Kendrick and Ratledge, 1992) was used in this study with few modifications. The optimized minimum medium contained (per liter of medium): 10 g glucose, 3 g or 0.3 g $(NH_4)_2SO_4$, 7 g KH_2PO_4, 2 g Na_2HPO_4, 1.5 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, 0.008 g $FeCl_3{\cdot}6H_2O$, 0.001 g $ZnSO_4{\cdot}7H_2O$, 0.0001 g $CuSO_4{\cdot}5H_2O$, 0.0001 g $Co(NO_3)_2{\cdot}6H_2O$, 0.0001 g $MnSO_4{\cdot}5H_2O$. The initial PH was adjusted to 5.0 by the addition of KOH. Cultures were initiated by inoculation of 2.5×10^5 spores/mL to a 50 mL Erlenmeyer flask containing 10 mL of medium and incubated at 30 °C with shaking at 250 rpm. *M. circinelloides* WJ11 and CBS 277.49 were grown in chemically defined media with different carbon-nitrogen (C/N) ratios. High carbon-nitrogen ratio (HN) medium contained (per liter of medium) 10 g glucose and 3 g $(NH_4)_2SO_4$, low carbon-nitrogen ratio (LN) medium contained 10 g glucose and 0.3 g $(NH_4)_2SO_4$.

Carbon isotope: D-[1-^{13}C] glucose and D-[U-^{13}C] glucose were purchased from Cambridge Isotope Laboratories Inc. Isotopic abundance was 99%.

Labeling experiment: the carbon source was 10 g [1-^{13}C] glucose/L or 2 g [U-^{13}C] glucose/L and 8 g unlabeled glucose/L.

2.2. Determination of physiological parameters

The samples were collected during the balanced growth phase. Biomass was harvested by filtration through a Buchner funnel under reduced pressure, washed three times with distilled water, and frozen at -80 °C. The frozen biomass was lyophilized and the CDW was determined gravimetrically. Glucose concentration in the culture was measured using Glucose oxidase Perid-test kit (Shanghai Rongsheng Biotech Co., Ltd) according to the manufactory's instructions. Ammonium concentration in the culture was determined using the indophenol method (Chaney and Marbach, 1962).

2.3. Lipid analysis

Approximately 20 mg of lyophilized biomass was used for fatty acid analysis, lipid was extracted with chloroform/methanol (2:1, v/v) (Folch et al., 1957), and pentadecanoic acid (15:0, Sigma) was added as an internal standard, then fatty acid methyl esters were formed by 10% HCl/methanol (w/w) and analyzed by gas chromatography (GC-2010; Shimadzu Co., Kyoto, Japan) with a DB-Waxetr column (30 m × 0.32 mm × 0.25 μm). The temperature program was as follows: 120 °C for 3 min, reached to190 °C at 5 °C per min, raised to 220 °C at 4 °C per min, and held for 20 min. Nitrogen was the carrier gas at a constant flow of 3 mL per min.

2.4. GC-MS analyses of protein hydrolysates

Approximately 50 mg of lyophilized biomass was hydrolyzed with 1.5 mL of 6 M HCl at 110 °C for 24 h. The hydrolysate was dried in an oven at 80 °C for 12 h and dissolved in 300 μL pyridine, then centrifuged at 12000 × g for 10 min. The supernatant was added with 50 μL N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (TBDMS, J & K Scientific Ltd.) and derivatized at 85 °C for 1 h. The derivatized samples were analyzed by GC-MS. GC-MS was carried out using an Agilent GC-6890 gas chromatograph equipped with an Agilent HP-5MS column (30 m × 0.25 mm × 0.25 μm) that was directly connected to an MS-5975 mass spectrometer (Agilent). The oven temperature was initially held at 60 °C for 2 min and reached 180 °C at 5 °C per min, then raised to 260 °C at 10 °C per min, and finally held at 260 °C for 5 min.

2.5. Metabolic network construction for M. circinelloides

The metabolic network of *M. circinelloides* was constructed based on genome characterization (http://genome.jgi.doe.gov/Mucci2/Mucci2.home.html) and genome-scale analysis of the metabolic networks of *M. alpina* and *M. circinelloides* (Wang et al., 2011; Vongsangnak et al., 2013). The constructed network models of *M. circinelloides* consist of the glycolytic pathway (EMP), the pentose phosphate pathway (PPP), the tricarboxylic acid cycle (TCA), the pyruvate/oxaloacetate/malate (POM) cycle and glyoxylate cycle (GOX).

2.6. Metabolic modeling and flux analysis

For metabolic flux ratio analysis, a mass isotopomer distribution vector of each amino-acid fragment MDV_α was assigned on the base of the well-developed mathematic methodology (Eq. (1)).