The Effect of Soybean Oil on Lipid Metabolism in *Mucor circinelloides* WJ11 by Metabolomic Analysis

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**Abstract:** Oleaginous fungus *Mucor circinelloides* utilizes different carbon sources associated with sustainable production of lipids, using soybean oil as carbon source, the fungus accumulated more intracellular lipids. Nonetheless, the metabolic changes in *M. circinelloides* upon vegetable oil as main carbon source have not been yet reported. Therefore, this study was conducted to investigate the metabolomics of *M. circinelloides* WJ11 cultivated in mixed glucose and soybean oil as carbon source and to reveal its effects on lipid metabolism. *M. circinelloides* strain WJ11 was cultured under optimized conditions in a fermenter and the biomass samples were collected after 24 h for lipid metabolite investigation. The frozen biomass samples were subjected to various chromatographic analyses like liquid chromatography and high resolution-mass spectrometry for metabolite profiling. A total of 438 differential metabolites were identified, of which 48 were up-regulated and 33 were down-regulated. Among them, lyso-phosphatidic acid and monoglyceride were up-regulated whereas phosphatidylglycerol and lyso-phosphatidylglycerol were down-regulated in the experimental group (soybean oil and glucose as mixed carbon sources) compared with the control group (glucose as single carbon source). Significant changes in metabolite levels correlated to lipid synthesis were identified. This study showed that the addition of soybean oil to the medium favors the triacylglycerol synthesis in *M. circinelloides*. Our results can also be applied to the investigation of other microorganisms and would contribute to further genetic engineering for higher lipid accumulation in fungi.

**Keywords:** *Mucor circinelloides*, Soybean Oil, Mixed Carbon Sources, Lipid Metabolomics, Lipid Molecules

**Introduction**

Oleaginous microorganisms accumulate lipids which may constitute more than 20% of their dry cell biomass having fatty acids composition almost similar to vegetable oil (Azócar et al., 2010). They can be cultivated under controlled conditions, utilizes inexpensive substrates and requires a limited space for their cultivation (Li et al., 2008). Lipids from microbial origin can be a possible alternative to plant oil and petro-based hydrocarbon sources. Oleaginous microbe’s uses a variety of low-cost carbon sources and are regarded as an effective and eco-friendly approach associated with sustainable production of lipids (Kamat et al., 2013). Several studies have been done on plant or vegetable oil as carbon source to replace glucose in microbial fermentation that showed change in production and composition of fatty acids in microbial lipids (Lim et al., 2001; Park and Ming, 2004; Tan and Ho, 1991; Darvishi et al., 2009). Among microbes various genera of filamentous fungi namely *Mucor*, *Rhizopus*, *Aspergillus*, *Fusarium*, *Penicillium* and *Geotrichum* has shown the ability to produce extracellular lipases that degrade triglycerides into free fatty acids (Colla et al., 2016). In fungi there are two mechanisms for lipid synthesis: Ex novo and de novo (Carsanba et al., 2018). In oleaginous fungi excess of carbon in the form hydrophilic substrates such as glucose in the culture media plays an important role in de-novo lipid synthesis (Wynn et al., 2001; Arous et al., 2015; Gujiala et al., 2019; Huang et al., 2018). While excess of carbon in the form of hydrophobic substrates such as cooking oils plays important role in ex-novo lipid synthesis.
biosynthesis (Subramaniam et al., 2010). Oleaginous fungi secrete extracellular lipase that hydrolyses oily substrate in medium into free fatty acids and the free fatty acids with the help of an active transport system are transferred inside the cells (Najjar et al., 2011; Carsanba et al., 2018). The high amount of free fatty acids inside the cell is biotransformed into new lipids by ex-novo synthesis (Probst et al., 2016; Beopoulos et al., 2009). M. circinelloides is useful as a model for the study of lipid metabolism and is also industrial strain used for long chain polyunsaturated fatty acid production (Ratledge and Wynn, 2002; Zhang et al., 2017). A typical oleaginous microorganism, M. circinelloides WJ11 accumulates over 36% lipids of their cell mass under normal fermentation conditions (Tang et al., 2015a). The genome of M. circinelloides WJ11, had been sequenced and comparative genomic approaches now provide an easy way to identify multiple genes that are expressed differentially (Tang et al., 2015b). Our previous work has demonstrated that M. circinelloides WJ11 contains many lipase genes (both intracellular and extracellular) and can use plant oil as carbon source (Zan et al., 2018). Lipases of M. circinelloides WJ11 grown in media with different carbon sources (glucose or soybean oil) have different mRNA levels. However, the mechanism of increased lipid accumulation in the fungus grown on oils has not been systematically investigated. Metabolomics generates a global profile of various biochemical metabolites of a biological system both quantitatively and qualitatively, that in turn reflects the activity of the metabolic network (Liu and Locasale, 2017). In this study, therefore, we investigated the mechanism of increased lipid accumulation in M. circinelloides grown in mixed glucose and soybean oil as carbon source by metabolomics analysis.

Materials and Methods

Materials

All the materials used in this study were commercially available. SPLASH internal standard stock solution (330707, SPLASHTM Lipidomix Mass Spec Standard, Avanti Polar Lipids, USA). Methanol (A454-4) and acetonitrile (A996-4) were liquid phase mass spectrometry (LC-MS) grade (Thermo Fisher Scientific, USA). All other reagents used in this study were analytical grade.

Experimental Methods

Strains Preparation

*Mucor circinelloides* WJ11 used in this experiment was preserved in Colin Ratledge Center for Microbial Lipids of Shandong University of Technology.

| Table 1: Preparation of seed medium and fermentation medium |
|-----------------------------------------------------------|
| Reagent | Seed medium | Fermentation medium |
|---------|--------------|---------------------|
| Glucose      | 30 g/L       | 80 g/L              |
| MgSO₄·7H₂O   | 1.5 g/L      | 1.5 g/L             |
| Ammonium tartrate | 3.3 g/L   | 2.0 g/L             |
| KH₂PO₄      | 7.0g/L       | 7.0g/L              |
| Na₂HPO₄     | 2.0 g/L      | 2.0 g/L             |
| Yeast extract | 1.5 g/L     | 1.5 g/L             |
| CaCl₂·2H₂O  | 0.1 g/L      | 0.1 g/L             |

Culture Conditions

*M. circinelloides* WJ11 was grown on nitrogen-limited K&R media (Kendrick and Ratledge, 1992). The major composition of the seed and fermentation medium is shown in the Table 1. In addition minor metal ion mixture needs to be added: 8 mg/L FeCl₂·6H₂O, 1 mg/L ZnSO₄·7H₂O, 0.1 mg/L CuSO₄·5H₂O, 0.1 mg/L Co(NO₃)₂·6H₂O and 0.1 mg/L MnSO₄·5H₂O. Treatment group used in this study was modified K&R medium prepared with mixed glucose (35 g/L) and soybean plant oil (24.3 g/L) as carbon sources.

Seed culture was prepared in a 1 L baffled flask by inoculating 100 μL of WJ11 spores (10⁶ spores/ml) into 250 mL medium in a K&R seed media. Cultures were incubated at 28°C for 24 h with shaking at 150 rpm. 10% (v/v) seed culture was used to inoculate 4 L fermenter containing 2.5 L modified K&R medium. Fermenters were controlled at 28°C with stirring at 700 rpm, aeration at 2 v/v/min⁻¹ and pH 6.

Extraction of Lipid Molecules

Based on growth and lipid accumulation characteristics, fermentation samples were collected at 24 h for lipid and metabolite analysis. Samples were filtered through the Buchner funnel to collect cell biomass. The biomass was immediately rinsed three to five times with PBS buffer and tapped in liquid nitrogen for 15 min. Then freeze samples were stored at -80°C for further experiments.

All samples were thawed slowly at 4°C, 25 mg of biomass was transferred into 1.5 mL Eppendorf tube. 800 μL of extraction solution (dichloromethane/methanol = 3:1, v:v, precooling at -20°C) and 10 μL of SPLASH internal standard stock solution were added into the tube. Two small steel balls were also added and put them into a tissue grinder for grinding (50 Hz, 5 min) and water bath ultrasound at 4°C for 10 min and then refrigerated at -20°C for 1 h. The samples were centrifuged at 25000 rpm at 4°C for 15 min. After centrifugation, 600 μL supernatant were collected, put it in the freeze vacuum concentrator to dry. 200 μL of reagent solution (isopropanol: Acetonitrile: H₂O = 2:1:1, v:v:v) were added for re-dissolution, vortexed vibration for 1 min. water bath ultrasound at 4°C for 10 min, then centrifuged
at 25000 rpm at 4°C for 15 min. 20 μL of the supernatant of each sample were taken and mixed it into a Quality Control (QC) sample to evaluate the repeatability and stability of the LC-MS analysis process.

Chromatographic Conditions

The column used was a CSH C18 column (1.7 μm 2.1×100 mm, Waters, USA). The positive ion mode mobile phases were aqueous solution containing 10 mM ammonia formate, 0.1% formic acid and 60% acetonitrile (liquid A) and a solution containing 10 mM ammonia formate, 0.1% formic acid, 90% isopropanol and 10% acetonitrile (liquid B). The negative ion mode mobile phases were aqueous solution containing 10 mM ammonia formate and 60% acetonitrile (liquid A) and 10 mM ammonia formate, 90% isopropanol and 10% acetonitrile (liquid B). The following gradients were used for elution: 0–2 min, 40–43% liquid B; 2–2.1 min, 43–50% liquid B; 2.1–7 min, 50–54% liquid B; 7–7.1 min, 54–70% liquid B; 7.1–13 min, 70–99% liquid B; 13–13.1 min, 99–40% liquid B, 13.1–15 min, 40% liquid B. The flow rate was 0.35 ml/min, the column temperature was 55°C and the injection volume was 5 μL.

Mass Spectrometry Conditions

Primary and secondary mass spectrometry data acquisition was performed using a Q Exactive mass spectrometer (Thermo Fisher Scientific, USA). The mass spectra scan mass to nucleus ratio range is 200–2000, with a primary resolution of 70,000, an AGC of 3e6 and the maximum injection time (IT, injection time) is 100 ms. The secondary information was acquired by selecting Top3 for fragmentation according to the parent ion intensity, with a secondary resolution of 17,500 and a secondary injection time (IT, injection time) is 50ms and the stepped nce is set to 15, 30, or 45 eV. The ion source (ESI) parameters were set: Sheath gas flow rate (40), Aux gas flow rate (10). Spray voltage (10). Spray voltage (Kv) is 3.80 for positive ion mode and 3.20 for negative ion mode. The ion transfer tube temperature (Capillary temp) is 320°C and the auxiliary gas heater temperature (Aux gas heater temp) is 350°C.

Data Processing and Statistical Analysis

In this study, the univariate analyses used were multiplicative analysis of variance change (Fold Change, FC) and Student's t-test. Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) technique was used for untargeted lipidomics analysis and a high-resolution mass spectrometer Q Exactive (Thermo Fisher Scientific, USA) was used to increase the lipid detection coverage by acquiring data in both positive and negative ion modes separately. LipidSearch 4.1 software was used for LC-MS/MS data processing, including a series of analyses for smart peak extraction, lipid identification and peak alignment. Statistical analysis was performed using the metabolomics R package metaX (Wen et al., 2017). The intergroup differences of the samples were observed by Principal Component Analysis (PCA) and the Variable Important in the Projection (VIP) values of the first two principal components of the Partial Least Squares method-Discriminant Analysis (PLS-DA) (Barker and Rayens, 2003; Westerhuis et al., 2008) model were used to screen the differential lipid molecules in combination with the multiplicative change of variance analysis and t-test.

Results

Growth and Lipid Accumulation

During our preliminary results it was observed that exogenous soybean oil as the carbon source affects growth and lipid accumulation of M. circinelloides WJ11. The biomass of the fungus grown in medium with soybean oil was higher as compared to glucose as sole carbon source. From the results of total lipid content analysis, it was observed that WJ11 grown in the medium contained soybean oil was up to 43.83%, increased by 17% compared to glucose (Table 2).

Base Peak Chromatogram

Intracellular extracts of M. circinelloides WJ11 grown in soybean oil (treated) and glucose (control) were subjected to lipid metabolomics profiling. To understand the central lipid metabolism of an oleaginous fungus upon catabolizing triglycerides and glucose as sole carbon source, all metabolites involved in lipid biosynthesis were targeted and quantified. The ion flow diagrams of the basal peaks of the samples were overlaid by in positive and negative ion modes. From the Base Peak Chromatogram (BPC), it can be seen that the chromatographic peak baseline is smooth and the retention time and peak response intensity fluctuates in control and treated group (Fig. 1) revealed that soybean oil substitution affects the cellular metabolisms.

Principal Component Analysis

A combination of multivariate statistical analysis and univariate analysis was used to screen metabolites that differed between groups. The multivariate statistical analysis methods used were PCA and PLS-DA. PCA analysis containing QC samples and all samples were used to observe the overall distribution in each group and the stability of the whole analysis process. PLS-DA can reflect the differences between categorical groups to the greatest extent. The method uses partial least squares regression to model the relationship between metabolite expression and sample categories to achieve modeling prediction of sample categories. The VIP was calculated to measure the strength and explanatory power of each metabolite expression.
pattern on the classification of each group of samples, thus assisting the screening of metabolic markers.

Table 2: Growth and lipid accumulation on glucose and oil mixed carbon sources medium

| Time(h) | Biomass Glucose(g/L) | Lipid content(w/w, lipid/biomass) Glucose(%) | Lipid content(w/w, lipid/biomass) Glucose/oil(%) |
|--------|----------------------|--------------------------------------------|-----------------------------------------------|
| 12     | 7.75 g/L             | 12.44 g/L                                  | 17.43                                         | 23.61                                         |
| 24     | 9.65 g/L             | 14.39 g/L                                  | 22.77                                         | 35.75                                         |
| 48     | 14.22 g/L            | 14.99 g/L                                  | 36.25                                         | 42.23                                         |
| 72     | 14.38 g/L            | 14.90 g/L                                  | 37.12                                         | 43.66                                         |
| 96     | 14.44 g/L            | 15.10 g/L                                  | 38.46                                         | 43.83                                         |

A

B

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PCA and PLS-DA analyses were first performed on the experimental (n = 5) and control (n = 5) samples to assess the statistical differences between the two groups. The PCA scores showed that the data points of the two groups showed significant spatial separation, indicating that there were differences in metabolic patterns between the two groups (Fig. 2A) and the PLS-DA analysis revealed that the data points of the experimental and control groups were clustered into one cluster, which showed that the soybean oil supplementation group was significantly different from the control group (Fig. 2B). The model parameters of the PLS-DA were $R^2 = 0.964$, $Q^2 = 0.956$. The results indicated that the model was not over-fitted and was robust enough to be used in the subsequent difference component analysis.
Fig. 2: PCA and PLS-DA scores of control and experimental groups when *M. circinelloides* WJ11 was grown in a mixed carbon source of soybean oil and glucose. (A) PCA model score. (B) PLS-DA model score. Red dots represent the control group and blue dots represent the experimental group with added soybean oil.

Fig. 3: The number of lipid subclasses and corresponding lipid molecules identified by MS analysis.
Fig. 4: Volcano plot of differential lipid molecules and variance multiplicity plot of significantly different lipid molecules. (A) Volcano plot. (B) Variance multiplicity plot. VIP greater than 1 indicates that the variable has a significant effect on the differentiation of sample categories. (LPA: Lyso-Phosphatidic acid, MG: Monoglyceride, PA: Phosphatidic Acid, OAHFA: (O-Acyl)-1-Hydroxy fatty acid, MGDG: Monogalactosyldiacylglycerol, PE: Phosphatidylethanolamine, PMe: Phosphatidymethanol, LPI: Lyso-Phosphatidylglinositol, PG: Phosphatidylglycerol, PI: Phosphatidylinositol, DG: Diglyceride, PS: Phosphatidylserine, TG: Triglyceride, PC: Phosphatidylcholine, LPG: Lyso-Phosphatidylglycerol)
Differential Metabolite Screening

Based on the mass spectrometry results, we counted the number of identified lipid subclasses and the corresponding lipid molecules (Fig. 3). From the results, we can see that the number of glycerophospholipids and glycerol esters among the lipid subclasses identified in this experiment was high.

Differential lipid molecules were visualized by volcano maps (Fig. 4A). The results showed that the addition of soybean oil to the culture medium did affect the metabolism of *M. circinelloides* WJ11. A total of 438 differential metabolites were identified. From the above differential metabolites, 81 metabolites with significant differences (VIP ≥ 1 for the first two principal components of the PLS-DA model, fold change ≥ 2 or ≤ 0.50, q-value < 0.05) were screened, of which 48 were up-regulated and 33 were down-regulated. These significantly different metabolites include phosphatidic acid (lyso-phosphatidic acid, phosphatidic acid, etc.), neutral glycerolipid (such as monoglyceride), fatty acid (such as (O-acetyl)-1-hydroxy fatty acid), P-glycerol (such as phosphatidylglycerol, lyso-phosphatidylglycerol). Among them, lyso-phosphatidic acid and monoglyceride were up-regulated and phosphatidylglycerol and lyso-phosphatidylglycerol were down-regulated in the treatment group compared with the control group. We visualized the fold change of the differential lipid molecules (only the first fifteen largest up- and down-regulation of the differential fold are shown) (Fig. 4B).

Discussion

The lipid metabolism analysis approach helped us to understand the effect of exogenous soybean oil on lipid accumulation in *M. circinelloides* WJ11 and the lipid metabolism analysis results showed that the phosphatidic acid content in the treated group of *M. circinelloides* WJ11 cells grown on medium supplemented with soybean oil was increased. Phosphatidic acid is an intermediate in the synthesis of triacylglycerols and glycerophospholipids. Our results are consistent with previous studies which showed that exogenous oil such as coconut oil, palm oil and other vegetable oils have positive effects on fungal biomass and lipid accumulation (Zan et al., 2018).

The differences in lipid metabolites suggested that the lipid metabolism of *M. circinelloides* WJ11 is altered when soybean oil is added to the medium as an additional carbon source. When soybean oil is present in the medium, *M. circinelloides* WJ11 secretes extracellular lipase to degrade the oil in the medium into small molecules of free fatty acids (Zan et al., 2018). When these free fatty acids enter into the cell, they can directly increase the lipid content in the cell. Secondly, these free fatty acids enter the cells to carry out *ex novo* synthesis of lipids. This process greatly improves the efficiency of intracellular lipid synthesis.

Our transcriptional analysis data showed that the gene expression of glycerol-3-phosphate O-acetyltransferase was up-regulated 23.39-fold in the cells of *M. circinelloides* WJ11 grown in soybean oil supplemented medium (unpublished data). This enzyme can catalyze the conversion of glycerol phosphate to lysophosphatidic acid, 1-acylglycerol-3-phosphate, which is a precursor substance for phosphatidic acid synthesis and the up-regulation of gene expression of this enzyme can explain the up-regulation of phosphatidic acid in lipid metabolism at the transcriptional level. Furthermore, the transcriptional analysis showed that the gene expression of 2-acylglycerolO-acetyltransferase, phospholipid: Diacylglycerol acyltransferase and diacylglycerol O-acyltransferase, which are involved in catalyzing the conversion of monoacylglycerol to diacylglycerol and then to triacylglycerol, were all up-regulated, which could also explain the increased lipid accumulation in *M. circinelloides* WJ11 grown on medium supplemented with soybean oil at the transcriptional level.

Conclusion

For the first time, by using a systematic metabolomics approach, we analyzed the metabolic change in lipid biosynthesis pathway of *M. circinelloides* WJ11 cultivated in the media with soybean oil as additional carbon source and revealed the effects of the soybean oil on lipid accumulation. The results showed that the addition of soybean oil to the medium alters the lipid accumulation of *M. circinelloides* WJ11 and favors the *ex novo* lipid biosynthesis. Metabolites that showed significant differences included phosphatidic acid, neutral glycerides and fatty acids etc. Among them, lyso-phosphatidic acid and monoglyceride were up-regulated, while phosphatidylglycerol and lyso-phosphatidylglycerol were down-regulated. This suggested that the metabolic shift in the fungus grown under soybean oil favors triacylglycerol synthesis.

This study provided some insights into the molecular mechanism of lipid accumulation in oleaginous fungi grown on plant oils, which is important for the development of microbial cell factories for lipid production using wasted or cheap oils and fats as substrate.

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Author’s Contributions

Caili Sun: Participated in the whole experiment process and also contributed to the interpretation of the results and manuscript preparation.

Aabid Manzoor Shah: Participated in the whole experiment process and also contributed to the manuscript preparation.

Junhuan Yang: Participated in part of the experimental design.

Chen Wu: Participated in part of the experiment.

Zongmin Wang: Ameliorated the manuscript.

Yuanda Song: Contributed to the guidance of experimental design and ameliorated the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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