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How nitrogen sources influence *Mortierella alpina* aging: From the lipid droplet proteome to the whole-cell proteome and metabolome

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ABSTRACT

Arachidonic acid (ARA) is a valuable polyunsaturated fatty acid produced by *Mortierella alpina*. Although some strategies such as nitrogen supplementation have shown the potential to affect the aging of *M. alpina* in ways which enable it to produce more ARA, the underlying mechanism remains elusive. Herein, we conducted a systematical analysis of the lipid droplet proteome, as well as the whole-cell proteome and metabolome, in order to elucidate how and why two different nitrogen sources (KNO_3 and urea) affect the aging of *M. alpina* and the corresponding ARA concentration. We found that KNO_3 promoted the ARA concentration, while urea accelerated lipid consumption and stimulated the decomposition of mycelia. Although both KNO_3 and urea activated carbohydrate metabolic pathways, KNO_3 exerted a stronger promoting effect on the pentose phosphate pathway and induced the lipid droplets to participate in the citrate-pyruvate cycle. The activities of malic enzyme and isocitrate dehydrogenase were also promoted more by KNO_3. These pathways provided additional substrates and reducing power for ARA synthesis and ROS elimination. Accordingly, since urea showed a weaker promotion of the related pathways, it caused a depression of the antioxidant system and a consequent increase of ROS. These findings facilitate the design of nitrogen supplementation strategies to achieve higher ARA concentrations, and provide guidance for deciphering the mechanisms of similar aging phenomena in other oleaginous microorganisms.

Significance: Polyunsaturated fatty acids such as arachidonic acid (ARA) are valuable nutrients, which play important roles in preventing numerous diseases and facilitating development. Although it has been found for years that ARA production will be increased in the aging process of *Mortierella alpina* (*M. alpina*) and nitrogen sources are involved in this process, the underlying mechanism for this phenomenon remains unknown. In this work, we used the subcellular proteomics, whole-cell proteomics and metabolomics methods to explore the mechanisms by which two different nitrogen (KNO_3 and urea) affected the aging process of *M. alpina*. Finally, we gave some new insights for the mechanisms mentioned above. This finding will fuel the technology developments for the ARA production using microbes.

1. Introduction

Aging is a common phenomenon in various species ranging from microbes to mammals and in many cases, it causes unwanted outcomes. However, the aging process of the filamentous fungus - *Mortierella alpina* (*M. alpina*) is particularly interesting and useful due to its influence on arachidonic acid (ARA) production [1,2]. Microbial oils can not only be used to produce biodiesel, but also serve as an important source of polyunsaturated fatty acids (PUFAs) such as ARA [1,3]. Due to the high physiological and therapeutic significance of ARA for humans, there is keen interest in the technical developments for producing microbial oils rich in ARA [1]. Culturing *M. alpina* in an environment without additional carbon sources after regular fermentation to induce aging is called aging technology and has been reported to be an economical and efficient way to improve the ARA concentration [1,4]. Thus, many efforts have been made to optimize the conditions for *M. alpina* aging,

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such as pH adjustment, temperature control, as well as controlled supplementation of nitrogen/carbon sources [4–6].

Nitrogen sources have important effects on cell growth and lipid accumulation in oleaginous microorganisms [7,8]. Moreover, a certain amount of nitrogen source is helpful for improving the activity of malic enzyme, which is an important NADPH provider in ARA synthesis [9]. Developing a smart nitrogen source supplementation strategy for aging technology will thus be beneficial for producing ARA in a more efficient and predictable way [1]. Unfortunately, as with many other biological approaches, the development of this strategy is hindered by our lack of detailed knowledge of the underlying mechanisms.

In the past decade, advancements in omics technologies have enabled the high-throughput monitoring of a variety of cellular events at the molecular level [10]. Since proteins and metabolites are direct regulators and participants of metabolic reactions, the combined analysis of the proteome and metabolome of a target microorganism enables a comprehensive understanding of fermentation processes, the underlying metabolic pathways as well as interaction networks [11]. In addition, recent studies have shown that lipid droplets (LDs) not only serve as an energy reservoir in oleaginous microorganisms, but also actively participate in many cellular processes such as autophagy and lipid metabolism via the proteins contained on their surface [12–15]. Because the LD proteome is intimately linked to other organelles and the whole-cell signalling pathways [13,16,17], running a parallel analysis of the lipid droplet proteome along with the whole-cell proteome and metabolome of an oleaginous microorganism will enable an in-depth understanding of important metabolic pathways and the role of LDs.

With regard to this, to fully understand the underlying mechanisms by which different nitrogen sources influence the aging process of *M. alpina*, we herein systematically investigated the changes of lipid accumulation, ARA percentage, and cellular morphology of cells aged with two different nitrogen sources - KNO_3 and urea. Furthermore, the whole-cell proteome, LD proteome and whole-cell metabolome were simultaneously investigated, and a systematic analysis allowed us to propose a mechanism guiding the aging process. Our findings will be useful for developing an optimal nitrogen source supplementation strategy based on aging technology to improve the ARA concentration. Furthermore, the results of this study can also guide the exploration of corresponding mechanisms in other oleaginous microorganisms in the future.

2. Materials and methods

2.1. Stains and culture conditions

M. alpina R807 (CCTCC M2012118) was obtained from the China Centre for Type Culture Collection and was cultured as reported previously [18,19]. Briefly, it was maintained on potato-dextrose agar (PDA) slants at 4 °C and transferred to fresh media every three months. The PDA medium comprised 25 g/L glucose, 20 g/L agar and 200 g/L potato extract. The seed medium were made of 30 g/L glucose, 3 g/L $NaNO_3$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 6 g/L yeast extract (Angel Company, China) and 3 g/L KH_2PO_4. The fermentation medium comprised 4 g/L KH_2PO_4, 3 g/L $NaNO_3$, 0.6 g/L $MgSO_4{\cdot}7H_2O$, 80 g/L glucose and 10 g/L yeast extract. Two square pieces (1 cm × 1 cm) of PDA slants covered with *M. alpina* mycelium were transferred into 100 mL of fresh media to inoculate the seed culture. Seed culture was conducted for one day, after which 10% (v/v) of the seed culture was used to inoculate the fermentation broth. After the regular fermentation, mycelia were further cultured without carbon source for the aging process. At the beginning of the aging process, KNO_3 or urea was added into individual fermentation broths to reach a final concentration of 1.6 g/L in each case. In the control group, no additional nitrogen source was supplemented. All cultivations were carried out at 25 °C, an initial pH 6.0 and 125 rpm shaking speed.

2.2. Analysis of dry cell weight, total lipids and fatty acid profiles

To measure the dry cell weight (DCW), mycelia were harvested and filtered through filter papers, washed three times, and dried to a constant weight (65 °C, 8 h). Lipid extraction and analysis was carried out using previously reported methods [20]. Briefly, the dried mycelia were ground into a powder in a mortar. Then, the lipids from 2 g of the resulting powder were extracted using 150 mL methanol/chloroform (v: v = 1: 2) in a Soxhlet extractor (8 h, 75 °C). A GC system (GC-2010, Shimadzu, Japan) equipped with a capillary column (DB-23, 60 m × 0.22 mm, Agilent, USA) and a flame ionization detector (FID) was utilized to analyze the fatty acids. The column was heated from 100 °C to 196 °C at 25 °C/min, and subsequently to 220 °C at 2 °C/min, where it was kept for 6 min. The temperature of the FID detector was set to 280 °C. N_2 was used as the carrier gas with a flow rate of 1 mL/min. The injector was maintained at 250 °C and the injection volume was 1 μL. ARA percentage (%) = ARA concentration (g/L)/lipid concentration (g/L) × 100%.

2.3. Observation of mycelial morphology and ultrastructure

In short, the mycelia were harvested by centrifugation and dispersed in glycerol to obtain a concentration of 0.1 g/mL. Three milliliters of the mycelia-glycerol suspension were supplemented with 5 μL Nile Red stock solution (0.4 mg/mL, J & K Scientific Ltd., China) and the resulting mixture was gently shaken for 1 min. After incubation for 10 min in darkness at room temperature, samples were observed under a confocal microscope (FV1000, Olympus, Japan). The excitation wavelength was set to 488 nm, and the range of emission wavelength scanning was 500–750 nm.

Mycelia were cut into about 5 mm pieces and that were prefixed in PBS (pH 7.4) containing 2.5% (w/v) glutaraldehyde at 4 °C for 24 h. Subsequently, samples were washed with PBS three times. The samples were then post-fixed with 2% (w/v) osmium tetraoxide (EM grade, Nakalai Tesque, Japan) at room temperature for 1 h. The samples were dewatered by subsequent rinses with solutions comprising 35%, 50%, 70%, 90%, 95%, and 100% ethanol (v/v). After being embedded in epoxy resin, the samples were cut into 90 nm sections using an ultramicrotome (EM UC6, Leica, Germany). The sections were further stained with uranyl acetate and lead citrate, and were observed under a transmission electron microscope (TEM) (JEM-1011, JEOL, Japan).

2.4. Lipid droplet isolation and lipid droplet proteome analysis

Lipid droplets (LDs) were isolated using the method reported by Ding et al. [21], with minor modifications as follows: Mycelia were harvested from 100 mL of culture broth, rinsed three times with PBS (pH 7.2), and resuspended in Buffer A (20 mM tricine, 250 mM sucrose, pH 7.8) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, USA). The resulting washed samples were placed on ice for 20 min and homogenized four times at 4 °C using a Nano Homogenizer (AH100B, Ats Engineering Inc., China) at 700 bar. The resulting homogenate was centrifuged at 3000 ×*g* for 10 min at 4 °C to remove unbroken cells, nuclei and cell debris. The resulting supernatant was designated the post-nuclear supernatant (PNS) fraction. Eight milliliters of the PNS fraction were moved into an SW40 tube and covered with 2 mL of buffer B (20 mM HEPES, 100 mM KCl, 2 mM $MgCl_2$, pH 7.4). The samples were further centrifuged at 182000 ×*g* for 1 h at 4 °C, and the floating LDs were transferred into an Eppendorf tube.

LD protein samples were separated by SDS-PAGE, after which the identified LD protein bands were excised from the gels. For protein digestion and removal of the low-molecular-weight components, the resulting gel slices were de-stained twice with 200 μL of acetonitrile – 25 mM ammonium bicarbonate (v: v = 2: 3) and rinsed three times with UA buffer (8 M Urea, 150 mM Tris-HCl, pH 8.0), followed by repeated ultrafiltration. 100 μL of 0.05 M iodoacetamide in UA buffer

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