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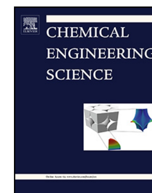

Manipulating the generation of reactive oxygen species through intermittent hypoxic stress for enhanced accumulation of arachidonic acid-rich lipids

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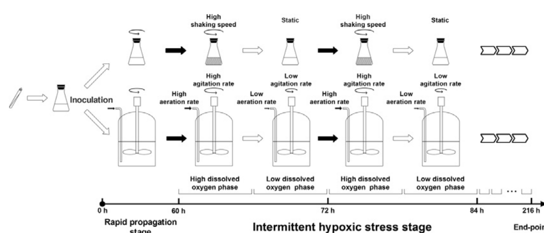
HIGHLIGHTS

- Intermittent hypoxia stress reduced ROS generation in *M. alpina*.
- ARA yield increased by about 20% along with a 21% decrease of ROS concentration.
- Biomass, lipid- and ARA concentration simultaneously reached their peak values at the end-point.
- Efficient ARA-rich lipids fermentation was fulfilled by using this strategy.
- The total stirring power of the fermentation was reduced by about 4% in a bioreactor.

GRAPHICAL ABSTRACT



Advantages:

(1) Arachidonic acid production was increased.

(2) Lipid production was increased.

(3) The total stirring power was reduced in a bioreactor.

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ABSTRACT

The accumulation of reactive oxygen species (ROS) can induce oxidative damage which can be detrimental to microbial biomass and product yields. In this study, we used intermittent hypoxic stress (IHS) to eliminate the main source of ROS during the production of arachidonic acid (ARA)-rich lipids by *Mortierella alpina*. Biomass, lipid- and ARA concentration simultaneously reached their peak values, and the ARA yield increased by about 20% along with a lower level of ROS concentration. Under these conditions, more NADPH, substrate and energy may be available for lipid biosynthesis. The efficacy of this strategy was also confirmed in a bioreactor, with similar results. Interestingly, the total power required for stirring fell by about 4%, which may be of practical value for energy savings. This work offers new insights into the control of ROS generation in *M. alpina* and might be applicable to other microbial PUFAs producers.

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1. Introduction

As essential components of the cells of higher eukaryotes, polyunsaturated fatty acids (PUFAs) confer flexibility, fluidity and selective permeability properties to membranes, and are indispensable for human health (Ratledge, 2004; Ryan et al., 2010;

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Ji et al., 2015). Arachidonic acid (ARA) (5, 8, 11, 14-*cis*-eicosatetraenoic acid) is one of the omega-6 PUFAs, and acts as a precursor for the synthesis of eicosanoid hormones such as prostaglandins, leukotrienes and thromboxanes, some of which play an irreplaceable role in combating or preventing a number of human diseases (Dyal and Narine, 2005). The filamentous fungus *Mortierella alpina* is considered the most prominent producer of ARA-rich lipids due to its safety and relatively high production (Higashiyama et al., 2002; Ji et al., 2014).

High dissolved oxygen (DO) is considered a key factor in the ARA biosynthesis process, as PUFAs are formed via an enzymatic desaturation mechanism which requires dioxygen (Nie et al., 2014b). In shake-flask cultures, the level of DO is controlled by changing the shaking speed. However, *M. alpina* is physically weak and sensitive to shear forces, due to which the agitation rate has to be controlled within a precise range (Ji et al., 2014). Furthermore, high DO is not always beneficial for lipid accumulation, and oxygen uptake is not rate-limiting at high oxygen concentrations. It has been reported that filamentous mycelia could not maintain their usual metabolic activity, and had to β-oxidize fatty acids to obtain more energy in order to adapt to high oxygen concentrations (Higashiyama et al., 1999). In addition, it is well established that reactive oxygen species (ROS), which are mainly generated by the electron transfer chain (ETC) in mitochondria, can cause oxidative damage to DNA, proteins, lipids, and other biomolecules (Starkov, 2008; Shi et al., 2017). Our previous research showed that ROS generation significantly increased and antioxidant defenses decreased during the end of fermentation, i.e. during the microbial aging process. Using transmission electron microscopy (TEM), we observed mitochondria that seemed to be enlarged and less compact than normal, which suggested instability, which in turn can cause ROS generation (Yu et al., 2016). Increased oxidative stress may lead to the consumption of stored lipids as energy source in order to maintain cellular homeostasis (Shi et al., 2017). Furthermore, PUFAs can be oxidized by ROS, which may cause unstable ARA production (Yu et al., 2016). In agreement with this, it has been reported in recent years that the external addition of antioxidants, such as sesamol, ginsenosides and ascorbic acid, can scavenge intracellular ROS and improve cell growth and lipid production in oleaginous microorganisms (Ren et al., 2017; Liu et al., 2015). Accordingly, different concentrations of ascorbic acid were supplemented to the fermentation medium in our preliminary experiments. However, no significant changes were found in ARA production, and we even observed a small decline (Table S1). As ME activity and G6PD activity were decline with the addition of ascorbic acid, the lack of NADPH supply may cause the unexpected results (Fig. S1).

In adaptive laboratory evolution, a recovery treatment performed after stimulation may help to ameliorate the increased toxicity due to environmental stresses (Reyes et al., 2014). In this work, we developed a similar strategy to eliminate ROS from the main source—the ETC. This strategy was applied repeatedly in a 12 h cycle after 60 h of fermentation. Each cycle was divided into two phases: a high-DO phase and a low-DO phase for intermittent hypoxic stress (IHS). Considering a predictable delay of cell growth, the agitation speed during the high-DO phase was set at 150 rpm (rpm), which was somewhat higher than the 125 rpm of the original culture. The ARA yield was improved by 20% or more, along with a 21% reduction of intracellular accumulated ROS. Various biochemical parameters like malonic dialdehyde (MDA), glutathione (GSH) and the activities of several key enzymes were evaluated to examine the responses of *M. alpina* to IHS. In addition, the efficacy of this strategy was also tested in a bioreactor and we reached similar results. Interestingly, the total power required for stirring was reduced by about 4%, which may be of practical value for energy savings. This study thus provides a new method for increasing ARA production by removing ROS in *M. alpina*, which also might be used in other microbial PUFA producers.

2. Materials and methods

2.1. Microorganism, media, and culture conditions

M. alpina R807 (CCTCC M2012118), preserved in the China Centre for Type Culture Collection, was used in this study. It was maintained on potato-dextrose agar (PDA) slants at 4 °C, and transferred every 3 months. The PDA medium contained ($g{\cdot}L^{-1}$): potatoes 200, glucose 25, agar 20. The seed culture medium contained ($g{\cdot}L^{-1}$): glucose 30, yeast extract 6, $NaNO_3$ 3, KH_2PO_4 3, $MgSO_4{\cdot}7H_2O$ 0.5. The fermentation medium contained ($g{\cdot}L^{-1}$): glucose 80, yeast extract 10, KH_2PO_4 4, $NaNO_3$ 3, and $MgSO_4{\cdot}7H_2O$ 1.

The culture conditions for *M. alpina* were the same as reported in our previous work (Zhang et al., 2015; Nie et al., 2014a; Peng et al., 2010). Briefly, a sterile loop was used to transfer a small amount of *M. alpina* mycelium from a PDA slant to fresh medium, followed by incubation in an electro-thermal incubator at 25 °C. After 5–7 days of incubation, the resulting mycelia were harvested for seed culture. 500-mL baffled flasks containing 100 mL of appropriate fresh media were used for seed culture and fermentation culture. Seed culture was conducted for 18–24 h at 25 °C, after which the resulting culture was used to inoculate the fermentation broth at 10% (v/v). After a course of regular fermentation, mycelia were continuously cultured without carbon source to initiate the aging process (Zhang et al., 2015). Shake-flask cultivation was carried out at 25 °C and 125 rpm, with an initial pH of 6.0. ARA batch fermentation was carried out in a 1-L bioreactor (Solaris Biotechnology, Italy) containing 0.6 L of fermentation medium, using a previously reported multi-stage fermentation strategy (Wu et al., 2017).

The procedures of the new IHS strategy are shown in Graphical abstract. This strategy was conducted repeatedly in a 12 h cycle after 60 h of fermentation. Each cycle was divided into two phases: a high-DO phase, and a low-DO phase for periodic hypoxia treatment. The DO of the high-DO phase was slightly above normal.

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

Glucose concentrations were analyzed using an SBA-40C glucose oxidase electrode (Biology Institute of Shandong Academy of Sciences, China). The biomass density was measured by determining the DCW of mycelia that were harvested and separated by filtration through a conventional filter paper, washed three times, and dried at 50 °C to constant weight (approx. 24 h). The analysis of total lipids and the fatty acid profiles was carried out according to our previously reported methods (Zhang et al., 2015). Polynomial fittings of the 4th order was used to describe the kinetics of DCW, lipid/DCW and ARA/total fatty acids (TFAs), using Origin 8.0 (Microcal Software Inc., Northampton, MA, USA).

2.3. Measurement of intracellular ROS

The contents of ROS in *M. alpina* mycelia was determined according to our previously reported methods (Yu et al., 2016). Briefly, the mycelia were harvested and incubated with 10 $\mu g\ mL^{-1}$ of DCFH-DA (Dichloro-dihydro-fluorescein diacetate, prepared in DMSO; Sigma-Aldrich, MO, USA) with shaking at 25 °C for 30 min, washed three times by centrifuging at 8000*g* for 10 min and resuspending in phosphate buffer solution (PBS, pH 7.4). The fluorescence density (FLU) was recorded using a SpectraMax M3 fluorescence spectrophotometer (Molecular Devices, CA, USA)

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