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Enhanced arachidonic acid production from *Mortierella alpina* combining atmospheric and room temperature plasma (ARTP) and diethyl sulfate treatments

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HIGHLIGHTS

- Novel mutagenesis technique with ARTP was performed for this study.
- An integrated and targeted method was established to select mutant strains.
- The relative content of ARA increased to 45.64%.
- The maximum of ARA yield reached 6.82 g/L.

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ABSTRACT

To obtain mutant strains with higher arachidonic acid (ARA) yields, the oleaginous fungus *Mortierella alpina* was mutated using atmospheric and room temperature plasma (ARTP) coupled with diethyl sulfate (DES). A visual compound filter operation was used in which a screening medium was supplemented with cerulenin, an inhibitor of fatty acid synthase (FAS), and triphenyltetrazolium chloride (TTC). The mutant strain D20 with an ARA production of 5.09 g/L, a 40.61% increase over the original strain (3.62 g/L), was isolated. The relative ARA content increased from 38.99% to 45.64% of total fatty acids. After optimizing fermentation conditions, the maximum ARA yield (6.82 g/L) for strain D20 was obtained in shake flasks. This work provides an appropriate strategy for obtaining high ARA-yield strains by conventional random mutation methods with an efficient screening assay.

1. Introduction

Arachidonic acid (ARA, C20:4) belongs to the ω-6 group of polyunsaturated fatty acids (PUFAs), which is increasingly in demand for its biological activity and clinical effects (Sakuradani et al., 2009; Nisha et al., 2011). Nowadays, ARA is widely used in biological medicine and in the chemical and food industries (Nie et al., 2014). For a long time, the conventional sources of ARA were mainly deep-sea fish, animal organs such as pig adrenal glands and egg yolks (Ward and Singh, 2005). However, low ARA contents and undesirable properties (such as poor flavor and seasonal restrictions) limit the use of these conventional sources for large-scale industrial production (Vadivelan and Venkateswaran, 2014). Therefore, alternative sources of ARA production must be sought. Previous studies have revealed that certain microorganisms can accumulate lipids, especially triacylglycerols (TAGs) (Liang and Jiang, 2013). Filamentous fungi belonging to the genus *Mortierella* have been identified as promising strains for ARA production, especially *Mortierella alpina*, which can accumulate substantial quantities of ARA in its mycelium (Yu et al., 2003). There are no reliable toxicology research reports showing toxic responses to this species or its products (Nisha et al., 2009). However, microbial lipids have not been widely commercialized due to factors such as low lipid productivity and unacceptable power consumption. More research is needed on the upstream and downstream portions of microbial lipid production (Ma et al., 2013).

Varying progress has been made to improve the ARA yield of *M. alpina* through metabolic engineering (Sakuradani et al., 2013). Recently, the mitochondrial ME (mME) gene of *M. alpina* was homologously over-expressed and the ARA content increased from 25% to 40% of total fatty acids (Hao et al., 2014). Molecular biology

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methods are widely used to directly modify desirable genetic characteristics. However, it is still under developed in the multiple gene expression system of *M. alpina*, presumably due to a lack of identification of the variations in promoters and a low transformation frequency (Mackenzie et al., 2000; Okuda et al., 2014). Furthermore, ARA biosynthesis is tightly controlled and highly regulated. The modification of one or two genes does not readily produce a transformant with the desired traits. Traditional mutation, such as by physical or chemical methods combined with a rational screening process, is still suitable for adapting a strain for cultivation to control costs or obtain increased yields of valuable metabolites (Li et al., 2013). Of the chemical methods, diethyl sulfate (DES) is a common alkylating agent used to induce high acetoin in *Bacillus subtilis* (Zhang et al., 2013). Atmospheric and room temperature plasma (ARTP) is a powerful and novel tool for physical microbial mutagenesis in microorganism breeding. ARTP driven by radio-frequency power can be generated at atmospheric pressure without a vacuum system and its plasma jet can be controlled at room temperature, which is more convenient for microbial mutation than the previously reported ion beam implantation (Liu et al., 2013; Fang et al., 2013).

In *M. alpina*, the first committed step in *de novo* fatty acid biosynthesis is the conversion of acetyl-CoA to malonyl-CoA catalyzed by acetyl-CoA carboxylase (ACC). Acetyl-CoA and malonyl-CoA are used in fatty acid biosynthesis to generate palmitic acid (C16:0), which is catalyzed by fatty acid synthase (FAS). A series of desaturation and elongation reactions are then carried out to form ARA. FAS is a multi-enzymatic complex known to be the key enzyme in lipid biosynthesis (Liang and Jiang, 2013; Hamid et al., 2010). In the fungus *Aspergillus oryzae*, a twofold increase in the accumulation of intracellular total fatty acids and triglycerides was obtained by the homologous overexpression of a FAS gene (Tamano et al., 2013). Cerulenin, isolated from the fungus *Cephalosporium caerulens*, was originally used as an antifungal antibiotic. It inhibits FAS activity by covalent modification of the active site (Johansson et al., 2008). Mutants demonstrating normal growth rates at a certain concentration of cerulenin are identified as having relatively high FAS activity and are good candidates for lipid production. Fatty acid desaturase can introduce a double bond into saturated or unsaturated fatty acyl-CoA substrates to produce PUFAs. Overexpression of the Δ5 desaturase led to a high production of ARA (from 35% to 40%) in the total fatty acids compared with the wild strain (Sakuradani et al., 2013). Thus, increased activity of fatty acid desaturase could result in a superior ARA content. Triphenyltetrazolium chloride (TTC) is an oxidant that can be reduced from colorless TTC to red triphenylformazan (TF). TTC is generally used to characterize desaturase activity by its color depth, allowing to isolate high ARA-containing mutants through their degree of staining.

The major challenge in acquiring mutants with the desired phenotype by random mutagenesis is the development of efficient screening methods to efficiently identify positive mutant strains (Tapia et al., 2012). In oleaginous microorganisms, especially filamentous fungi, previous screening methods have used random selection based on low temperature stress or microscopic observation after staining with liposoluble dyes. However, an efficient screening method must be able to target a large number of individual mutants. A screening filter that can easily indicate target characteristics is very important for improving screening efficiency. In the present work, novel ARTP irradiation-induced mutation combined with DES treatment was carried out on the wild-type *M. alpina* to improve the quality and production of ARA. A simple targeted screening strategy based on cerulenin and TTC in a two-step mutation-isolation process was carried out. In addition, the fermentation condition of the mutant was optimized, including the nitrogen source and fed-batch culture conditions.

2. Methods

2.1. Microorganism and culture conditions

The initial strain, *M. alpina*, was isolated and preserved in our laboratory. The mature spores of wild-type *M. alpina* in slants were eluted with sterile water. The spores were broken into single spores by 3–4 mm glass beads and filtered through three layers of sterile lens paper to obtain the spore suspension. One milliliter of spore suspension (1×10^8 cells/mL) was inoculated into a seed medium containing (g/L): glucose 30.0, yeast extract 30.0, KH_2PO_4 0.2 and $MgSO_4{\cdot}7H_2O$ 1.0 at pH 6.0. The inoculums were prepared in 250 mL flasks containing 50 mL medium. The culture was grown for 36 h at 25 °C on a shaker rotating at 200 rpm. Then, 250 mL flasks containing 50 mL production medium were inoculated at 10% (v/v) and incubated on a shaker at 25 °C and 200 rpm. The production medium contained (g/L): glucose 50.0, yeast extract 25.0, KH_2PO_4 0.2 and $MgSO_4{\cdot}7H_2O$ 1.0 at pH 6.5. All of the media were sterilized at 115 °C for 20 min. The glucose solution was sterilized separately.

Different fed-batch operations were used to optimize the fermentation conditions as described in Section 3. During cultivation, aliquots of 500 g/L glucose stock were prepared and added to the medium once a day, giving a feed of 10 g/L glucose. The effects of different types of nitrogen sources were evaluated at equivalent total nitrogen concentrations of 2.5 g/L. The nitrogen sources tested included sodium nitrate, potassium nitrate, ammonium sulfate, ammonium chloride, tryptone, urea, soy flour and yeast extract. Compound nitrogen sources were also evaluated.

2.2. Mutagenesis by ARTP and rational screening with cerulenin

The ARTP mutation was implemented at Wuxi Siqingyuan Biotechnology Co., Ltd. Pure helium was used as the plasma working gas in the ARTP mutation and the operating parameters were as follows: (1) the radio frequency power input was 100 W; (2) the distance between the plasma torch nozzle exit and the sample plate was 2 mm; and (3) the temperature of the plasma jet was below 30 °C. Under these operating conditions, the ARTP mutagenesis dosage was only dependent on the treatment time. To determine the optimal treatment period, 20 μL fresh spore suspension (1×10^6 cells/mL) was evenly spread on a sterilized steel plate and exposed to the ARTP jet for 150, 165, 180, 195, 210 and 225 s, respectively. After each treatment, the dry mycoderm was eluted with sterile distilled water into a new tube and properly diluted, then grown on a PDA (potato dextrose agar) medium for 3 days at 25 °C prior to determination of the lethality rate. The individual colonies on the control medium and each mutated medium were counted, respectively. The lethality rate was determined as follows:

$$\text{Lethality rate } (\%) = \frac{\text{control colonies} - \text{survival colonies}}{\text{control colonies}} \times 100\%$$

For the first round of screening after ARTP mutagenesis, the PDA medium was supplemented with various cerulenin concentrations (0, 1.79, 1.88, 1.97, 2.06, 2.15, 2.24, 2.33 and 2.42 μM). A spore suspension of the wild-type strain was incubated on these plates at 25 °C for 3 days to achieve the proper concentration of cerulenin for pre-selection. After determining the optimal concentration, the irradiated spores were properly diluted and grown on a PDA medium with cerulenin for 3 days at 25 °C. Relatively large colonies were selected and transferred to a fresh medium for re-screening in shake flasks. Using this screening method, one mutant strain with highest ARA yield was used for the next DES mutation.