Caucasian Journal of Science
ISSN: 2148-6840
https://doi.org/10.48138/cjo.1559402
Year: 2024
Volume:11 Issue: 2
To Cite: Vardar Yel N., Yelboğa E., Karagüler N. & Tüter M. (2024). Enhancement of Docosahexaenoic Acid Production by UV Mutagenesis Coupled with Flow Cytometry Screening in Schizochytrium sp. S31. Caucasian Journal of Science, 11(2), 105-116

UV Mutagenez ve Akış Sitometrisi Taraması ile *Schizochytrium* sp. S31'de Dokosahekzaenoik Asit Üretiminin Artırılması

Enhancement of Docosahexaenoic Acid Production by UV Mutagenesis Coupled with Flow Cytometry Screening in *Schizochytrium* sp. S31

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Biyoloji / Biology **Araştırma Makalesi / Research Article**

Makale Bilgileri

Geliş Tarihi
01.10.2024
Kabul Tarihi
24.12.2024
Anahtar Kelimeler
Schizochytrium sp. S31, dokosaheksaenoik asit, rastgele mutajenez, akış sitometrisi

Öz

Mikroalgler, omega-3 yağ asitleri de dahil olmak üzere zengin biyoaktif bileşikleri nedeniyle terapötik ve farmakolojik uygulamalardaki potansiyelleri nedeniyle büyük ilgi görmüştür. Bunlar arasında, *Schizochytrium* sp. bu değerli lipitleri yüksek seviyelerde üretme kabiliyeti nedeniyle kapsamlı bir şekilde incelenmiştir. Bu çalışmanın amacı, genomda UV kaynaklı rastgele mutasyonlar oluşturarak *Schizochytrium* sp. S31 mutant kütüphanesi oluşturmak ve ardından akış sitometrisi tabanlı teknoloji kullanarak yüksek lipid birikimine sahip mutantları taramaktır. *Schizochytrium* sp. S31'in yüksek verimli lipid biriktiren mutantlarını izole etmek için rastgele mutagenez ve akış sitometrisi tabanlı seçimin bir kombinasyonu kullanılmıştır. Sonuçlar, Mutant 1'in toplam lipid içeriğinde %28,4'lük bir artış sergilediğini, Mutant 2'nin ise yabani tipe göre %10,8'lik bir artış gösterdiğini ortaya koymuştur. Sonuçlar, UV ışığı (30 saniye boyunca) ile muamele edilen kültürlerin, muamele edilmeyen kontrollerden daha yüksek DHA seviyeleri sergilediğini gösteren gaz kromatografisi-kütle spektrometresi ile desteklenmiştir. DHA yüzdesi, yabani tipe göre iki farklı mutantta %17,9 ve %12,1 oranında artmıştır.

Article Info

Received
01.10.2024
Accepted
24.12.2024
Keywords
Schizochytrium sp. S31, docosahexaenoic acid, random mutagenesis, flow cytometry

Abstract

Microalgae have garnered significant attention for their potential in therapeutic and pharmacological applications due to their rich bioactive compounds, including omega-3 fatty acids. Among these, *Schizochytrium* sp. has been extensively studied for its ability to produce high levels of these valuable lipids. The aim of this study was to create a *Schizochytrium* sp. S31 mutant library by generating UV-induced random mutations in the genome and then screening for mutants with high lipid accumulation using flow cytometry-based technology. A combination of random mutagenesis and flow cytometry-based selection was employed to isolate high-yield lipid-accumulating mutants of *Schizochytrium* sp. S31. The results revealed that Mutant 1 exhibited a 28.4% increase in total lipid content, while Mutant 2 demonstrated a 10.8% increase relative to the wild type. The results were corroborated by gas chromatography-mass spectrometry, which indicated that the cultures treated with UV light (for 30 seconds) exhibited higher levels of DHA than the untreated controls. The percentage of DHA increased by 17.9% and 12.1% in two distinct mutants relative to the wild type.

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1. INTRODUCTION

Microalgae represent a highly diverse unicellular group found in various ecosystems across the Earth, primarily in oceans and freshwater bodies. It is estimated that there are over 50,000 species of microalgae, yet only a limited number have undergone analysis. One such species is *Schizochytrium sp. S31*, a marine thraustochytrid known for its lipid-rich content, particularly in polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) (Yokochi et al., 1998). This microalga contains a range of bioactive compounds with high-value applications, including their use as pharmaceutical raw materials, food additives, and supplements (Koyande et al., 2019; Sathasivam et al., 2017; Kay & Barton, 1991; Pulz & Gross, 2004; Garcia et al., 2017). Moreover, microalgae serve a vital role in biofuel production, offering an alternative energy source, and supporting aquaculture practices (Spolaore et al., 2006; Chisti, 2007; Brennan & Owende, 2010). Extensive research has been conducted to characterize the lipid content of various algal species. Notably, microalgal lipid content exhibits substantial variation, ranging from 5% to 70% of dry weight biomass across different species (Mata et al., 2010; Brown et al., 1997; Han et al., 2011). The major fatty acids in most microalgae cells are palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2) or α-linolenic acid (C18:3) (Zhukova & Aizdaicher, 1995; Servel et al., 1994; Viso & Marty, 1993). Certain microalgal species possess the capability to synthesize long-chain polyunsaturated fatty acids (LC-PUFAs), including omega-3 and omega-6 fatty acids (Adarme-Vega et al., 2014; Khozin-Goldberg et al., 2011; Ryckebosch et al., 2012; Lenihan-Geels et al., 2013; Gupta et al., 2012; Raghukumar, 2008). These species hold significance due to their nutritional advantages (Ryckebosch et al., 2012; Unkefer et al., 2017; Doughman et al., 2007; Deckelbaum & Torrejon, 2012; Adarme-Vega et al., 2012). Research has demonstrated variations in lipid composition among microalgal species, with microalgae generally exhibiting a higher content of neutral lipids compared to other species (Lv et al., 2010). The composition and fatty acid profile of lipids, both saturated and polyunsaturated, have been influenced by factors including the life cycle of microalgae and various physicochemical conditions. These conditions encompass parameters such as temperature, pH, aeration rate, medium composition, stress conditions, and illumination intensity (Guzman et al., 2010; Rao et al., 2007; Wu et al., 2013). Numerous studies have reported the capacity of UV mutagenesis to enhance lipid content in microalgae (Bougaran et al., 2012; Liu et al., 2015; Manandhar-Shrestha & Hildebrand, 2013; Lim et al., 2015; Lian et al., 2010). In one particular study, UV mutagenesis was applied to augment

neutral lipid productivity in *Microalgae Isochrysis Affinis Galbana* (T-Iso) through a mutation-selection procedure (Bougaran et al., 2012). Another notable study achieved a 33% increase in both EPA and DHA levels in *Pavlova lutheri* through the implementation of UV mutagenesis (Meireles et al., 2003). Additionally, the EPA content of *Phaeodactylum tricornutum* experienced a 37% increase through the same technique (Alonso et al., 1996). The combination of UV mutagenesis with fluorescence-activated cell sorting (FACS) has emerged as a widely employed method for isolating potential mutants with enhanced lipid content in microalgae, without compromising cellular growth. This integrated approach enables high-throughput screening to identify the cells exhibiting the highest lipid production, utilizing Nile red luminescence as a basis for selection (Dempster & Sommerfeld, 1998). The use of UV mutagenesis in conjunction with FACS has been documented in several studies, encompassing high lipid-producing *I.galbana* strains and carotenoid hyperproducing *D. salina* strains (Bougaran et al., 2012; Mendoza et al., 2008).

2. MATERIALS AND METHODS

2.1. Microorganism and growth medium

A stock culture of Schizochytrium sp. S31 (ATCC 20888) was maintained using F/2 medium. Large-scale growth experiments were conducted employing both complex and fermenter media.

2.2. Death curve analysis

The experiment's initial phase involved inducing mutations in a wild-type Schizochytrium sp. culture using UV radiation from a lamp emitting a wavelength of 250 nm, positioned 7 cm above the culture. The samples consisted of 5 mL cultures with a cell density of 106 cells/mL. To assess cell viability, the culture was divided into five separate petri dishes, each subjected to UV exposure for 5, 15, 30, 45, and 60 seconds, respectively. To prevent photoreactivation, the samples were kept in darkness for 24 hours. After appropriate dilution, the treated cultures were plated, and the resulting colonies were collected and counted. The UV exposure duration that resulted in a 50% survival rate was determined to be optimal for mutant development, with untreated cells serving as a control.

2.3. Construction of mutant library

The mutant library was constructed following the methodology detailed by Manandhar-Shrestha and Hildebrand (2013). The Schizochytrium sp. S31 cell culture was grown to the early exponential phase before being harvested. The resulting cell pellet was washed and resuspended in F/2 medium to achieve a concentration of 3x106 cells/mL. These cells were then placed in a sterile 60 mm petri dish, covered with two layers of Kimwipe, and exposed to UV light (UVP CX-2000) for 30 seconds at maximum intensity (1 joule/cm2) from a distance of 7 cm. To prevent photoreactivation, the samples were kept in darkness overnight following exposure. The mutant cultures were subsequently transferred into both liquid and agar growth media and incubated for 2-3 days at 25°C. The purified cell pellet was then inoculated into NP medium and cultured for 24 hours at a temperature range of 20-25°C to induce lipid production.

2.4. Staining by fluorescent dye BODIPY

The lipophilic fluorescent dye BODIPY (4,4-difluoro-3a,4a-diaza-s-indacene), sourced from Invitrogen, was employed for the detection of intracellular lipids in Schizochytrium sp. The cell culture was first washed and then resuspended in a 0.1 M potassium phosphate buffer at pH 7. Subsequently, the cells were stained with BODIPY at a concentration of 2.6 µg/mL for 20 minutes at room temperature, followed by placement on ice. The constructed and induced mutant library was then screened using fluorescence-activated cell sorting (FACS) to identify and isolate the desired mutants.

2.5. Fluorescent activated cell sorting (FACS)

The mutant library was screened using high-throughput Fluorescence Activated Cell Sorting (FACS). Cells were labeled with BODIPY at a concentration of approximately 106 cells/mL. The sorting process was carried out using a Becton Dickinson Influx flow cytometer, excited by a 488-nm laser. Cells exhibiting elevated BODIPY fluorescence, indicative of high lipid content, were selectively collected from the upper fraction of the population. A total of 100,000 cells were sorted and then transferred to a liquid medium containing antibiotics for further growth.

2.6. Image Stream analysis

The quantification of TAG accumulation was conducted by means of BODIPY fluorescence using an ImageStreamX imaging flow cytometer (Amnis Corp., Seattle, WA). A total of $1x10^7$ cells were extracted from the culture at various time points during the incubation process and subjected to staining with BODIPY for lipid analysis. Cell classifier settings were configured to capture images with an area falling within the range of 50 µm2 to 300 µm2. Specifically, the bright-field image was recorded in channel 4, the side scatter image in channel 6 (with an excitation wavelength of 785 nm and emission range of 745-800 nm), BODIPY fluorescence in channel 2 (with an excitation wavelength of 488 nm and emission range of 470-560 nm), and chlorophyll autofluorescence in channel 5 (with an excitation wavelength of 488 nm and emission range of 660-720 nm).The 488 nm laser was adjusted to a power of 15 mW, while the bright-field 785 nm laser was set to 2 mW. The magnification level was established at 40X. For data analysis, the core velocity value was configured at 66, and the core track was adjusted to accommodate bead fluids. Data collection encompassed 5,000 to 10,000 cells for each analysis.

2.7. Gas Chromatography Analysis

The FAME (Fatty Acid Methyl Ester) analysis was conducted following the established procedure as outlined by Schlechtriem et al. (2008). To initiate the process, the total lipid content was suspended in a solution consisting of 5 mL of 0.5 N methanolic NaOH and subjected to heating at 100°C for a duration of 10 minutes. Subsequently, 5 mL of BF3-methanol was introduced for methylation purposes. The resulting FAMEs were extracted from the upper phase using 5 mL of n-heptane, followed by solvent evaporation and the addition of NaCl. To remove any residual moisture, the FAMEs were transferred to a vial containing Na2SO4 and heptane. These prepared samples were then subjected to analysis utilizing an Agilent 6850 Gas Chromatograph (GC) equipped with an Agilent HP 88 column (0.25 mm, 100 m, 0.2 µm). The detection and quantification of FAMEs were accomplished through chromatographic comparison with established standards provided by Sigma Chemical Co. in the USA.

3. RESULTS

3.1. Cellular Growth and Lipid Accumulation

The study demonstrated a time-dependent relationship in cell survival rates. Cell enumeration on agar plates revealed that a 30-second exposure to 1 joule of UV radiation resulted in a 50% survival rate, as shown in Figure 1. To evaluate differences in lipid accumulation, 100,000 cells were screened using FACS. Phosphorus and nitrogen deficiencies led to a significant rise in the mean BODIPY fluorescence value (530/40) [488] in 48 hours of incubation (Figure 2).

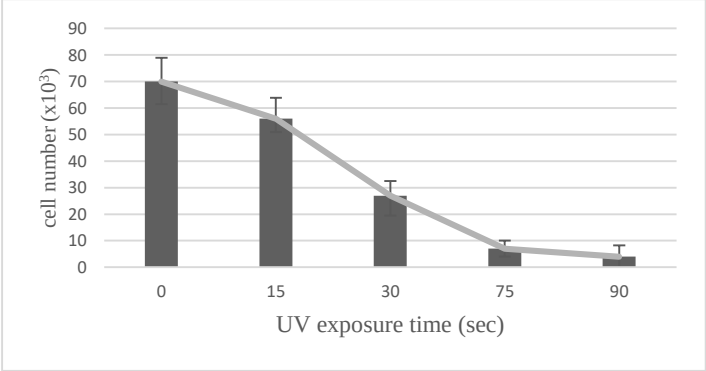

Figure 1. Schizochytrium sp. death curve under different UV exposure time

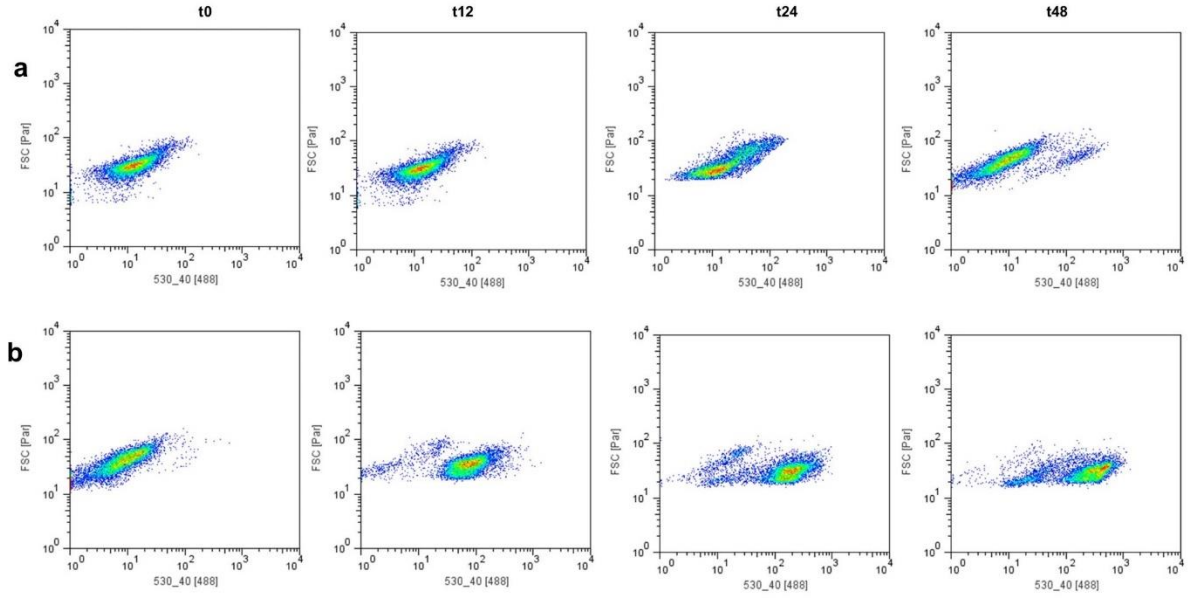

Figure 2. Flow cytometry analysis of the Schizochytrium sp. cell during the time under normal and limited conditions. a: Growth medium; b: Phosphorus and nitrogen limited medium

3.2. UV Mutagenesis and Screening

After three rounds of sorting and plating on agar, six mutant colonies which showed high lipid ratio signal in FACS results and one wild-type colony were selected for ImageStream (IS) analysis, with 50,000 cells sorted per treatment. IS analysis showed that two mutants (M1 and M2) exposed to 30 seconds of UV radiation had higher lipid content than the wild type.

Further IS analysis was performed on two mutants and the wild type, each with two replicates, to assess variations in fluorescent intensity over time. Mutant 1 and Mutant 2 exhibited significantly higher fluorescence levels than the wild type, with increases of 63.2% and 52.1%, respectively, as shown in Figure 3. A total of 5,000 cells were analyzed to compare the mutants with the wild type, revealing marked differences in fluorescent intensity.

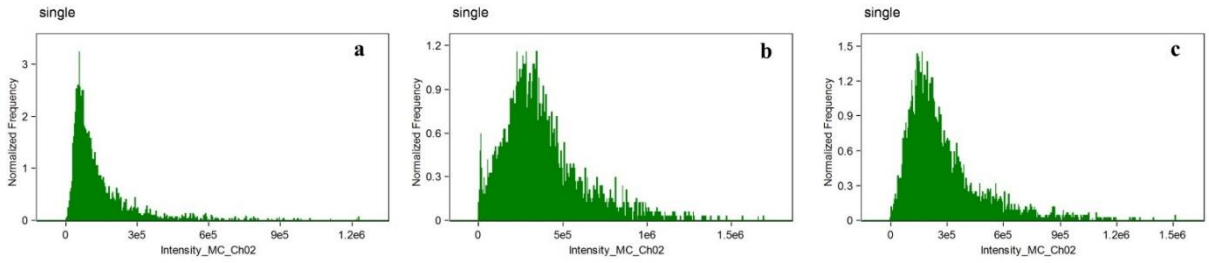

Figure 3. Light intensity difference between mutants and wild type on IS analysis a: Control; b: Mutant 1; c: Mutant 2 (30 sec UV exposure)

3.3. Characterization of Selected Mutants

The selected mutants were characterized by comparing parameters such as cell dry weight (CDW), total lipid content, DHA percentage, biomass productivity, and lipid productivity to the wild type, as detailed in Table 1. All tests were conducted in 1-liter baffled Erlenmeyer flasks under consistent conditions and were replicated twice. The wild-type CDW was 34.1 g/L, while Mutant 1 and Mutant 2 had CDWs of 28.5 g/L and 31.4 g/L, respectively. The total lipid content for the mutants consistently exceeded that of the wild type, with values of 25.6% for Mutant 1, 22.1% for Mutant 2, and 19.93% for the wild type.

GC-MS analysis was performed to assess fatty acid composition, revealing DHA concentrations of 156 mg/L in the wild type, 198 mg/L in Mutant 1, and 178 mg/L in Mutant 2, as shown in Table 1. This analysis confirmed the flow cytometry results, indicating a 17.9% increase in DHA for Mutant 1 and a 12.1% increase for Mutant 2 compared to the wild type

Table 1. Comparative results of the Mutant 1, Mutant 2 and Wt *Schizochytrium* sp.

	Mutant1	Mutant2	Wild type
%lipid	25,6	22,1	19,93
lipid productivity (g/l.h)	0,0729	0,0693	0,0679
%DHA	27,13	25,79	23
DHA productivity (g/l.h)	0,0198	0,0179	0,0156

4. DISCUSSION

UV mutagenesis is a well-established method for inducing lipid accumulation in various microalgal species (Tale et al., 2018; Trovão et al., 2022; Banerjee et al., 2018). Previous studies, such as Bougaran et al. (2012), demonstrated that combining UV mutagenesis with fluorescence-activated cell sorting (FACS) in Isochrysis galbana resulted in an 80% increase in lipid productivity (Bougaran et al., 2012). Similarly, the present study employs UV mutagenesis coupled with high-throughput selection using ImageStream to enhance lipid production in Schizochytrium sp. microalgae. Under nutrient-deprived conditions, microalgal cells typically shift their lipid biosynthesis pathways, accumulating neutral lipids—mainly in the form of triacylglycerol (TAG)—as a protective mechanism to endure environmental stress (Minhas et al., 2016). Studies have shown that temperature and nitrogen limitation maximize lipid content in microalgal cultures (Rios et al., 2015; Jiang & Chen, 2000a; Jiang & Chen, 2000b).

In this study, nitrogen (N) and phosphorus (P) starvation increased the mean BODIPY fluorescence value (530/40 [488]) from 78 to 290, indicating enhanced lipid accumulation. UV treatment of Schizochytrium sp. cells for 30 seconds led to faster lipid accumulation in mutants compared to the wild type (WT), as confirmed by time-course experiments. The total lipid content was 19.93%, 25.6%, and 22.1% for WT, Mutant 1, and Mutant 2, respectively. This corresponds to a 28.4% increase in Mutant 1 and a 10.8% increase in Mutant 2 relative to the wild type.

Further analysis via GC-MS revealed that UV-treated mutants also showed higher levels of docosahexaenoic acid (DHA), with Mutant 1 and Mutant 2 displaying increases of 17.9% and 12.1%, respectively, compared to the WT. These results are consistent with findings from Meireles et al. (2003), who observed a 33% increase in both eicosapentaenoic acid (EPA)

and DHA in Pavlova lutheri following UV mutagenesis (Meireles et al., 2003). Similar techniques have been successfully applied to enhance EPA content in Phaeodactylum tricornutum by 37% (Alonso et al., 1996). However, the effects of UV mutagenesis can vary across species, as differential stress tolerance mechanisms influence lipid accumulation outcomes (Gao et al., 2009). In the current study, further UV-induced mutations did not result in additional lipid content increases beyond the initial mutation trials, suggesting that the limits of lipid productivity under these specific stress conditions had been reached. These findings emphasize the importance of optimizing mutagenesis parameters and stress conditions for each species.

5. CONCLUSION

This study demonstrates the efficacy of UV mutagenesis combined with high-throughput selection techniques in enhancing lipid productivity in *Schizochytrium* sp. microalgae. Mutant 1 exhibited a 28.4% increase in total lipid content, while Mutant 2 showed a 10.8% increase compared to the wild type. GC-MS analysis corroborated the flow cytometry results, revealing significant elevations in DHA levels in UV-treated cultures, with a 17.9% and 12.1% increase in Mutant 1 and Mutant 2, respectively. These results suggest that nutrient deprivation and UV exposure act synergistically to boost lipid accumulation, likely as a cellular adaptation to stress. However, additional mutations beyond the initial trials did not yield further improvements, indicating that lipid enhancement through mutagenesis may have species-specific limitations

Acknowledgement

This research was supported by Scientific and Technological Research Council of Turkey (TUBITAK) with the International Research Fellowship for PhD Students (2214-A). The author grateful to Prof. Dr. Mark Hildebrand, for his great guidance and support while performing research at Scripps Institution of Oceanography-UCSD.

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