Journal of Bioscience and Bioengineering
VOL. 118 No. 6, 632–639, 2014

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# Extraction of squalene as value-added product from the residual biomass of *Schizochytrium mangrovei* PQ6 during biodiesel producing process

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Received 27 January 2014; accepted 20 May 2014
Available online 25 June 2014

**Today microalgae represent a viable alternative source of squalene for commercial application. The species *Schizochytrium mangrovei*, a heterotrophic microalga, has been widely studied and provides a high amount of squalene, polyunsaturated fatty acids and has good profiles for biodiesel production. Our work was aimed at examining the squalene contents in Vietnam's heterotrophic marine microalga *S. mangrovei* PQ6 biomass and residues of the biodiesel process from this strain. Thin-layer chromatography and high-performance liquid chromatography (HPLC) methods were successfully applied to the determination of squalene in *S. mangrovei* PQ6. The squalene content and production of *S. mangrovei* PQ6 reached 33.00 ± 0.02 and 33.04 ± 0.03 mg $g^{-1}$ of dry cell weight; and 0.992 g $L^{-1}$ and 1.019 g $L^{-1}$ in 30 and 150 L bioreactors, respectively after 96 h of fermentation. In addition, squalene was also detected in spent biomass (approximately 80.10 ± 0.03 mg $g^{-1}$ of spent biomass) from the *S. mangrovei* PQ6 biodiesel production process. The structure of squalene in residues of the biodiesel process was confirmed from its nuclear magnetic resonance spectra. The results obtained from our work suggest that there is tremendous potential in the exploitation of squalene as a value-added by-product besides biodiesel from *S. mangrovei* PQ6 to reduce biodiesel price.**



[**Key words:** Biodiesel; Hydrocarbon; *Schizochytrium mangrovei* PQ6; Squalene; Value-added co-product]

In the midst of the world energy crisis, third-generation biofuels (derived from algae) have been considered to be viable fuel alternatives (1). Among many types of algae, microalgae seem to be promising because of that they have high growth rate, e.g., doubling in 24 h. Their lipid content could be adjusted through changing growth medium composition (2,3). They could be harvested more than one in a year. Salty or wastewater could be used for their cultivation. They do not compete with traditional agriculture because they are not traditional foods and they can be cultivated in large open ponds or in closed photobioreactors located on nonarable land. They can grow in a wide variety of climate and water conditions (4). Moreover, microalgae can utilize and sequester $CO_2$ from many sources (5). Biodiesel from algal lipid is non-toxic and highly biodegradable and microalgae produce 15–300 times more oil for biodiesel production than traditional crops on an area basis (6,7). However, so far, biofuels derived from microalgae are not economically competitive. Therefore, it is crucial to explore approaches to reduce the costs of algal biodiesel production processes, by using low-cost raw materials and/or coproducing high value-added products. A few experimental works have been published using microalgae to obtain biofuel and high value-added products, within this concept (1,8). The valuable co-products present in the microalgal biomass mainly referred are polyunsaturated fatty acids (PUFAs), omega-3 fatty acids, fertilizers, plastics (e.g., polyhydroxyalkanoates, PHAs), recombinant proteins, pigments and hydrocarbon, such as squalene.

Squalene (2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaene) is one of the components of olive oil and can impact human health. It is an unsaponifiable lipid containing six isoprene units that provide the backbone for the biosynthesis of cholesterol, bile acids and steroid. Moreover, squalene is also a basic intermediary metabolite for the biosynthesis of sterols and triterpenes in plants and animals (9). Recent epidemiological studies have indicated that squalene can effectively inhibit chemically-induced lung, colon and skin tumorigenesis in animals under experimental conditions (10). It can protect cells against free radicals, strengthen the body's immune system, and decrease the risk for various cancers and lower cholesterol levels. Clinical studies have demonstrated that 60–85% of the total amount of squalene from dietary sources is effectively absorbed and distributed to various tissues. Higher squalene intake (around 500 mg per day) appears to be vital for maintaining nutritional health of human beings (11).

Currently commercial sources of squalene are often limited to liver oils of deep-sea sharks and plant seed oils (12). However, continuous supply and future availability of these sources are

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http://dx.doi.org/10.1016/j.jbiosc.2014.05.015

uncertain because of concerns over the preservation of marine wildlife as well as the effects of locality and seasonal variation on crop production (13). In addition, the use of shark liver oil is limited due to the presence of environmental pollutants such as dioxins, polychlorinated biphenyls and heavy metals in shark liver, as well as the unpleasant fishy smell and taste (14,15). As a result, many efforts have been made by scientists and researchers to find alternative squalene sources that have the potential to meet commercial production of high-quality squalene. Production of polyunsaturated fatty acids from oleaginous microorganisms rather than from marine animals such as fish and seals has now been made possible (16). However, currently microbial squalene sources have not been produced enough squalene for commercial applications.

In recent studies, microalgae have been explored as an alternative source of squalene (17,18). The green microalga *Botryococcus braunii* is capable of synthesizing squalene, but the very low growth rate and the obligate autotrophic growth characteristic render it unsuitable for commercial production. Of all the microalgal groups, the heterotrophically-grown thraustochytrids are regarded as a promising cell factory for the production of high-value products such as squalene (19,20). Thraustochytrids can growth rapidly in heterotrophic conditions when supplied with organic carbon. They are considered light-independent because they lack the photosynthetic apparatus for carbon fixation (21). Some thraustochytrids contain high squalene content such as *Thraustochytrium* ACEM 6063 (0.1 mg $g^{-1}$ of biomass) and *Aurantiochytrium mangrovei* FB1 (0.162 mg $g^{-1}$ of biomass) (22,23). The study of Fan et al. (13) demonstrated the production potential of squalene by *A. mangrovei* FB3. The highest cellular squalene content (0.53 mg $g^{-1}$) was achieved in culture supplemented with a glucose concentration of 30 g $L^{-1}$ and 100 mg $L^{-1}$ of terbinafine (terbinafine is an inhibitor of squalene monooxygenase in the sterol biosynthetic pathway). This value was much higher than those previously reported in *Saccharomyces cerevisiae* (0.041 mg $g^{-1}$ of biomass) and *Torulaspora debrueckii* (0.24 mg $g^{-1}$ of biomass) (24). Nakazawa et al. (25) reported that when the strain of *Aurantiochytrium* sp. 18W-13a was grown in the optimum condition (25°C, 25–50% seawater concentration and 2–6% glucose concentration), the squalene content and production of approximately 171 mg $g^{-1}$ of dry cell weight and 0.9 g $L^{-1}$ were much higher than those previously reported in thraustochytrids, plants and yeasts.

The production potential of squalene by thraustochytrid *Schizochytrium mangrovei* PQ6 has been investigated in our laboratory. This microalga was also highlighted the possibility to produce profitable biodiesel as well as the high-value PUFAs (8). Squalene is a non-fuel product extracted from *S. mangrovei* PQ6. Therefore, studies on how to obtain squalene as another value-added co-product of biodiesel production can shed lights on potential methods to reduce the cost of biodiesel.

In this study, the determination of squalene from Vietnam's heterotrophic marine microalga *S. mangrovei* PQ6 biomass and residues of the biodiesel process from this strain were developed. The squalene as a value-added product from biodiesel spent biomass achieved from our obtained results is one of the promising option for lowering the biodiesel cost.

## MATERIALS AND METHODS

**Algal strain and culture conditions** In this work, we used the microalga *S. mangrovei* PQ6 which was isolated from Phu Quoc Island, Kien Giang province, Vietnam (accession number SPQKG02) and later deposited at the Department of Algal Biotechnology, Institute of Biotechnology belonging to Vietnam Academy of Science and Technology, Vietnam. Fermentation was carried out using a fermentor/bioreactor (New Brunswick Scientific Co. Inc., Edison, NJ, USA) of two different volumes (30 and 150 L with working volume of 15 and 100 L, respectively), and a M12 medium that contained 90 g $L^{-1}$ glucose, 10 g $L^{-1}$ yeast extract, and 17.5 g $L^{-1}$ artificial seawater (ASW). The inoculum size was 2–3% of the total liquid volume of the bioreactor. Temperature was kept at 28°C. Dissolved oxygen was maintained at above 10% by manually increasing the stirring speed (Rushton blade impellers) from 250 rpm to a maximum of 450 rpm. The aeration rate was kept constant at 0.5 vol air (vol medium)$^{-1}$ $min^{-1}$ after filtering through a 0.2 μm filter. The medium pH was controlled within the range of 6.5–7.5. Addition of antifoam was not necessary during the fermentation. Upon reaching maximum biomass and lipid content, PQ6 biomass was harvested after 96 h of fermentation. In PQ6 strain, there was correlation between biomass and cellular lipid content as described in the report of Hong et al. (26). Algal biomass was harvested by centrifugation at 4000 rpm for 10 min. The algae paste was washed three times with sterile distilled water, dried to a constant weight in an oven at 80°C and then stored in desiccators. To eliminate batch-to-batch variation, all biomass was grown under the same conditions and thoroughly mixed to ensure a homogeneous biomass stock.

**Cell growth** Cell growth was determined by dry weight and cell density as described in the report of Hong et al. (26).

**Nile Red staining** *S. mangrovei* cells were stained with 15 mM Nile Red [(9-(Diethylamino)-5H-benzo [α] phenoxazin 5-one)] obtained from Sigma–Aldrich (USA) using protocol of Jara et al. (27). A working solution (50 μL) of Nile Red and acetone (0.1 mg $mL^{-1}$) was added to 1 mL of cell suspension. This mixture was gently vortexed and incubated for 10 min at room temperature in darkness. The stained microalgal cells were observed by using fluorescent microscopy (Nikon eclipse 80i-Japan). The excitation and emission wavelength were 480 and 575 nm, respectively (excitation and emission slits at 5 nm), according to the method recommended by Elsey et al. (28). The epifluorescent images were obtained at 1500× magnification.

**Preparation of squalene from biomass of *S. mangrovei* PQ6** The dried biomass of *S. mangrovei* PQ6 was used for squalene extraction follow two steps process. The first step was lipid extraction. The second step was to remove the saponifiables lipid from total lipid.

Analysis of total lipid content was determined as described in the report of Bligh and Dyer (29). Dried biomass (1 g) was placed in a chamber and 5 mL of distilled water was added to the biomass. 6 mL of chloroform and 12 mL of methanol were then added to the chamber. The mixture was homogenized well for 5 min and transferred to a centrifuge tube. Chloroform (6 mL) were used to rinse the chamber and then transferred to the tube, which was thoroughly mixed for 60 s. Distilled water (6 mL) were added to the tube followed by mixing in the same manner. The mixture was centrifuged at 6000 rpm for 10 min. The organic solvent layer containing the algal lipid was then collected. The solvent was evaporated to dryness in a water bath at 60°C. The dried residue was defined as the total extracted lipid. The weight of this residue was recorded for a gravimetric estimate of total yield.

Total lipid was placed in a Pyrex flask and mixed with a solution of 5% (w/v) potassium hydroxide in methanol–water (4:1 v/v). The reaction mixture was heated and maintained at 60°C for 3 h and well mixed throughout the experiment. After the reaction, following a cool down time, 4 mL of distilled water were added. The nonsaponifiable lipids were extracted three times with a mixture of hexane-chloroform (4:1 v/v) (10 mL each time). The entire nonsaponifiable lipids in *n*-hexane phase were combined and the solvent was evaporated to dryness under nitrogen

**TABLE 1.** Changes of parameters of *S. mangrovei* PQ6 in 30 L and 150 L bioreactors.

| Culture time (h) | Cell density (×$10^6$ cells $mL^{-1}$) | | Dry cell weight (g $L^{-1}$) | | Total lipid (% dry cell weight) | | Squalene (mg $g^{-1}$ dry cell weight) | |
|---|---|---|---|---|---|---|---|---|
| | 30 L | 150 L | 30 L | 150 L | 30 L | 150 L | 30 L | 150 L |
| 0 | 1.56 ± 0.04 | 1.56 ± 0.04 | – | – | – | – | – | – |
| 8 | 16.54 ± 0.21 | 23.49 ± 0.17 | 4.14 ± 0.04 | 5.88 ± 0.06 | 3.18 ± 0.02 | 4.52 ± 0.09 | 1.72 ± 0.03 | 1.87 ± 0.05 |
| 24 | 50.12 ± 0.12 | 61.20 ± 0.22 | 12.13 ± 0.04 | 14.81 ± 0.05 | 13.66 ± 0.03 | 16.68 ± 0.08 | 6.36 ± 0.05 | 6.50 ± 0.03 |
| 48 | 70.40 ± 0.64 | 78.49 ± 0.76 | 22.01 ± 0.12 | 24.54 ± 0.27 | 42.95 ± 0.11 | 47.89 ± 0.16 | 17.12 ± 0.05 | 17.61 ± 0.03 |
| 72 | 85.28 ± 0.07 | 88.22 ± 0.06 | 26.58 ± 0.05 | 27.50 ± 0.15 | 43.74 ± 0.09 | 45.25 ± 0.12 | 26.02 ± 0.03 | 25.14 ± 0.04 |
| 96 | 124.14 ± 0.21 | 127.45 ± 0.35 | 30.05 ± 0.16 | 30.85 ± 0.67 | 50.46 ± 0.08 | 51.80 ± 0.08 | 33.00 ± 0.02 | 33.04 ± 0.03 |
| 120 | 124.12 ± 0.13 | 126.23 ± 0.24 | 30.03 ± 0.11 | 30.26 ± 0.35 | 50.03 ± 0.09 | 51.06 ± 0.09 | 32.43 ± 0.04 | 32.65 ± 0.03 |

–, not determined. Value are expressed as mean ± SD ($n = 5$).