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OPTIMIZATION OF CULTURE CONDITIONS FOR SQUALENE PRODUCTION AND SQUALENE EXTRACTION METHOD OF *Thraustochytrium* sp. TN22

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ABSTRACT

Squalene, a natural triterpene with important roles as antioxidant, skin hydrating, cardio-protection and detoxifier, has attracted the attention of researchers in the world. This study investigated the optimal culture conditions for squalene production and to develop a method for squalene extraction and purification from cell suspension of *Thraustochytrium* sp. TN22. The results showed that squalene production by the strain *Thraustochytrium* sp. TN22 was optimum at 2% glucose, 0.5% yeast extract and 0,14% mixture of vitamins (B_1, B_6 and B_{12}) at 28 ^oC for 2 days of cultivation. At the optimum conditions, the dry biomass, squalene content and productivity were approximately 9.3 g/L, 9.9 mg/g DCW and 95.3 mg/L, respectively. Time-dependence on cell lysis and extraction solvents were selected as the extraction parameters. The obtained results showed that the highest squalene production of 178.1 mg/100 g biomass was obtained in cell lysate at alkaline medium (pH = 10), at 60 ^oC for 6 hours and n-hexan was the best solvent for squalene extraction. Squalene was then purified on silica gel column using n-hexan as the mobile phase and 90% purity of squalene was archived. Our obtained results are promising for the commercial productions of various value-added compounds from species belong to the genus *Thrautochytrium* in Vietnam.

Keywords: *Thraustochytrium*, culture, isolation, squalene, production.

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INTRODUCTION

Squalene is a natural lipid belonging to the terpenoid family and can impact human health. The most common source of squalene is liver oil of deep-sea sharks and whales. Sources for squalene production (primarily in vegetable oils) are identified in amaranth seed, rice bran, wheat germ and olives. All plants and animals produce squalene as a biochemical intermediate or precursor for biosynthesis of steroid hormones in eukaryotic cells, plants, animals and human (Pollier et al., 2019). Squalene has wide applications ranging from cosmetic industry to medical and pharmaceutical sector, owing to its broad functionalities. Furthermore, squalene is also found to exhibit radioprotective and cardioprotective activities (Spanova & Daum, 2011). Additionally, squalene is also a value-added product besides algal biodiesel (Hoang et al., 2014). The global demand for squalene has been increasing over the past decade, amounting to around 4,000 tons in 2019 (Tran et al., 2020), and could not be met solely by extracting it from the liver of marine animals; an approach that is severely affecting marine ecosystems. Plant oils are known to be capable of producing adequate quantities of squalene for pharmaceutical or nutraceutical industrial applications (Popa et al., 2015). However, recent researches suggested that thraustochytrids could serve also for large-scale commercial generation of squalene (Xie et al., 2017).

Thraustochytrid are heterotrophic marine oleaginous microorganisms capable of synthesizing high amounts of DHA, as well as other nutraceutical compounds such as squalene in their cellular compartment (Hoang et al., 2018; Patel et al., 2019). It is reported that the squalene contents in species belonging to thraustochytrids range from 0.18 to 84 mg/g of dry cell weight (DCW) (Li et al., 2009; Hoang et al., 2014; Otagiri et al., 2017; Hoang et al., 2018; Patel et al., 2019). Some thraustochytrids contain a high content of squalene, such as *Thraustochytrid* ACEM 6063 (0.1 mg/g of biomass), *Aurantiochytrium mangrovei* FB 1 (0.162 mg/g of biomass), *Schizochytrium mangrovei* PQ6 (98.07 mg/g of lipid), *Aurantiochytrium* sp. 18W-3a (171 mg/g DCW) and *Aurantiochytrium* sp. (318 mg/g DCW) (Patel et al., 2019). In Vietnam, *Thraustochytrium* sp. strain TN22 was successful isolated from Thi Nai marsh, Binh Dinh Province, Vietnam (Hoang Lan Anh et al., 2010). Results of morphological characteristic and molecular analysis showed that *Thraustochytrium* sp. TN22 belongs to genus *Thraustochytrium*, but it is not yet known exactly which species it belongs to. *Thraustochytrium* sp. TN22 grew well in Bajpai medium with cell density and dry cell weight (DCW) of 241.44×10^6 cells/mL and 4.79 g/L, respectively after 5 days of cultivation. The total lipid content and essential PUFAs, such as docosahexaenoic acid (DHA, C22: 6n-3), docosapentaenoic acid (DPA, C22: 5n-6) and eicosapentaenoic acid (EPA, C20: 5n-3) of the strain were 5.06% of fresh weight, 35.17%, 12.18% and 1.097% of total fatty acid, respectively, after 4 days of cultivation in 1 liter flask. In addition, the strain of TN22 showed to accumulate significant amount of carotenoid (up to 5.2 mg/kg of DCW) and squalene (up to 22 mg/g of DCW) (Hoang Lan Anh et al., 2010; Dinh Thi Ngoc Mai et al., 2013). Jiang et al. (2004) reported that the squalene level almost depends on the culture conditions. In present study, the optimal conditions for high squalene production of *Thraustochytrium* sp. TN22 were investigated. Additionally, the methods for extraction and purification of squalene from cell suspension of this strain were developed.

MATERIALS AND METHODS

The strain and inoculation

The microalgae *Thraustochytrium* sp. strain TN22 isolated from Thi Nai, Binh Dinh,

Vietnam (with accession number TTNBD02) which was deposited at Department of Algal Biotechnology, Institute of Biotechnology, Vietnam Academy of Science and Technology was used in this study. This strain was kept at 25–28 ^{o}C on glucose-peptone-yeast extract (GPY) medium which contained glucose 3 g/L, polypeptone 2 g/L, yeast extract 1 g/L, agar 15 g/L, and artificial seawater (ASW) 17.5 g/L as described by Hoang Thi Lan Anh et al. (2010). A single colony cultivated on a plate was picked up and transferred to a 250 mL Erlenmeyer flask containing 100 mL of the modified Bajpai medium (Bajpai et a., 1991; Hoang et al., 2010) (NaCl 25 g/L, $MgSO_4.7H_2O$ 5 g/L, KCl 1 g/L, KH_2PO_4 0.1 g/L, $CaCO_3$ 0.2 g/L, $(NH_4)_2SO_4$ 0.2 g/L, sodium glutamate 2 g/L, $NaHCO_3$ 0.1 g/L, glucose 20 g/L, yeast extract 10 g/L) and incubated for 4 days at 28 ^{o}C with shaking at 150 rpm for the preparation of a seed culture. The 4 mL of the seed culture were then transferred to 500 mL Erlenmeyer flasks containing 200 mL of the modified Bajpai and incubated at 28 ^{o}C with continuous shaking at 200 rpm.

Optimization of culture conditions

The growth and squalene production of *Thraustochytrium* sp. TN22 were investigated under four different parameters (carbon sources, glucose concentration, yeast extract concentrations and vitamin mixture). For carbon sources, cells were growth in the modified Bajpai medium with glucose, maltose, or starch at concentration of 2%. To test the effect of glucose concentrations, cells were growth in the modified Bajpai medium with glucose in different concentrations of 1%, 2%, 3% and 4%. To investigate the effect of yeast extract concentration, the cells were grown in M12 basal medium with 0.25%, 5%, 1% and 1.5% yeast extract. To perform the effect of the addition of vitamins, the cells were grown in M12 basal medium with and without supplementary addition of 0.14% vitamin mixture (vitamin B_1 45 g/L, vitamin B_6 45 g/L and vitamin B_{12} 0.25 g/L). Each parameter was tested separately with other parameters was kept constant. The culture conditions with the greatest growth were statistically validated and set as the optimal conditions. Cells were harvested after 1, 2, 3, and 5 days of cultivation by centrifuging at 3000 rpm for 10 min and keep at (-)20 ^{o}C until use for growth and squalene accumulation analysis.

Analytical methods under optimum conditions

Cell growth was determined by measuring DCW as described in our previous study (Dang Diem Hong & Hoang Thi Lan Anh, 2016).

The dried biomass of *Thraustochytrium* sp. TN22 cultured in the optimal culture conditions was used for squalene extraction following to two steps process. The first step was lipid extraction as in the report of Bligh and Dyer (1956) with some modifications as described by Hoang et al. (2014). The second step was to remove the saponifiable lipid from total lipid. Briefly, 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 ^{o}C for 3 hours and well mixed throughout the experiment. After the reaction, following a cool down time, 4 mL of distilled water was added. The unsaponifiable lipid was extracted three times with a mixture of n-hexane-chloroform (4:1, v/v) (10 mL each time). The entire unsaponifiable lipid in n-hexane layer was combined and the solvent was evaporated to dryness under nitrogen gas. Squalene was separated from unsaponifiable lipid by thin–layer chromatography and analyzed as described by Hoang et al. (2014).

Extration of squalene from cell suspensions of *Thraustochytrium* sp. TN22

The cell suspensions of *Thraustochytrium* sp. TN22 was removed from the Erlenmeyer flask, and then concentrated to 100 g/L by settling for 2 hours and removing the medium.

The biomass was kept stirring at 150 rpm in a 200 mL Erlenmeyer flask and heated to 60 ^{o}C. The pH was then adjusted to 10 with 45% potassium hydroxide. These conditions were kept for 1, 2, 4, 6 and 8 h in order to achieve complete alkaline lysis. The quality of the lysis was monitored under a light microscopy (Olympus CX21, Tokyo, Japan) and by sample centrifugation (2 min, 10,000 g). At the end of lysis, one volume of ethanol was added to the flask maintained at 45 ^{o}C and stirred for 10 min. One volume of n-hexane; methanol: chloroform (2:1, v/v); or petroleum ether was then added to the flask and kept stirring for 30 min. The mixture was then centrifuged in order to separate the solvent fraction which was stored in the other Erlenmeyer flask. The aqueous fraction was again brought together with one volume of above organic solvents so as to perform a second extraction according to the same scheme as previously in order to increase the extraction yield. The solvent fractions were combined and evaporate in a rotary evaporator (70 ^{o}C). Squalene was then separated from extracted unsaponifiable lipid by thin-layer chromatography and analyzed as described by Hoang et al. (2014).

Purification and structural identification of squalene

The crude squalene were further purified by column chromatography on a silica gel 60 (24 g, 70e230 mesh ASTM, EMD Millipore, MA, USA) column. A solution of 0.2 g of the crude squalene in 5 mL of n-hexane was loaded and eluted with n-hexane at a flow rate of approximately 1.0 mL/min. Test tubes (10 mL with screw cap) were used for fraction collecting and thin layer chromatography (TLC) was used for squalene detection. The fractions containing squalene were combined and evaporated up to dryness in vacuum condition by vacuum evaporator to obtain the purified squalene.

The structure of purified squalene was confirmed by the nuclear magnetic resonance (NMR) spectroscopy. NMR experiments were performed using a Bruker Avance e 500 MHz spectrometer (Bruker, Karlsruhe, Germany) at operating frequencies of 500 MHz (^{1}H) and 125 MHz (^{13}C) at the Institute of Chemistry, VAST, Vietnam.

Statistics

The data were analysed by a one-way ANOVA and a Tukey-Kramer post-hoc analysis was used to detect significant differences between the means at a level of $P < 0.05$.

RESULTS AND DISCUSSION

Optimization of culture conditions

The effects of various culture conditions such as carbon sources, glucose concentration, yeast extract concentrations and vitamin mixture on the cell growth, lipid and squalene accumulation of *Thraustochytrium* sp. TN22 were examined.

Carbon source test

It is acknowledged that *Thraustochytrium* sp. was able to grow on maltose, soluble starch (starch), and glucose; by contrast, fructose and sucrose did not support the growth (Shene et al., 2019). According to Nakazawa et al. (2012), Jiang et al. (2004), Chen et al. (2010), Patel et al. (2019), squalene content of thraustochytrids was the highest at 2% glucose but production per liter was not significantly different at concentrations from 2% to 6%. Therefore, glucose, maltose and starch at concentration of 2% were used to investigate the effect of carbon sources on cell growth and squalene production of the strain TN22 in this study (Fig. 1).

Figure 1 showed that when glucose was use as carbon sources, high levels of DCW and squalene content were obtained. Carbon sources such as maltose and starch were less effective for both of cell growth and squalene accumulation. The maximum DCW was reached at 8.6 g/L on day 3th, while squalene content and production reached maximum value of 5.9 mg/g of DCW and 44.7 mg/L on second cultivation day, respectively.

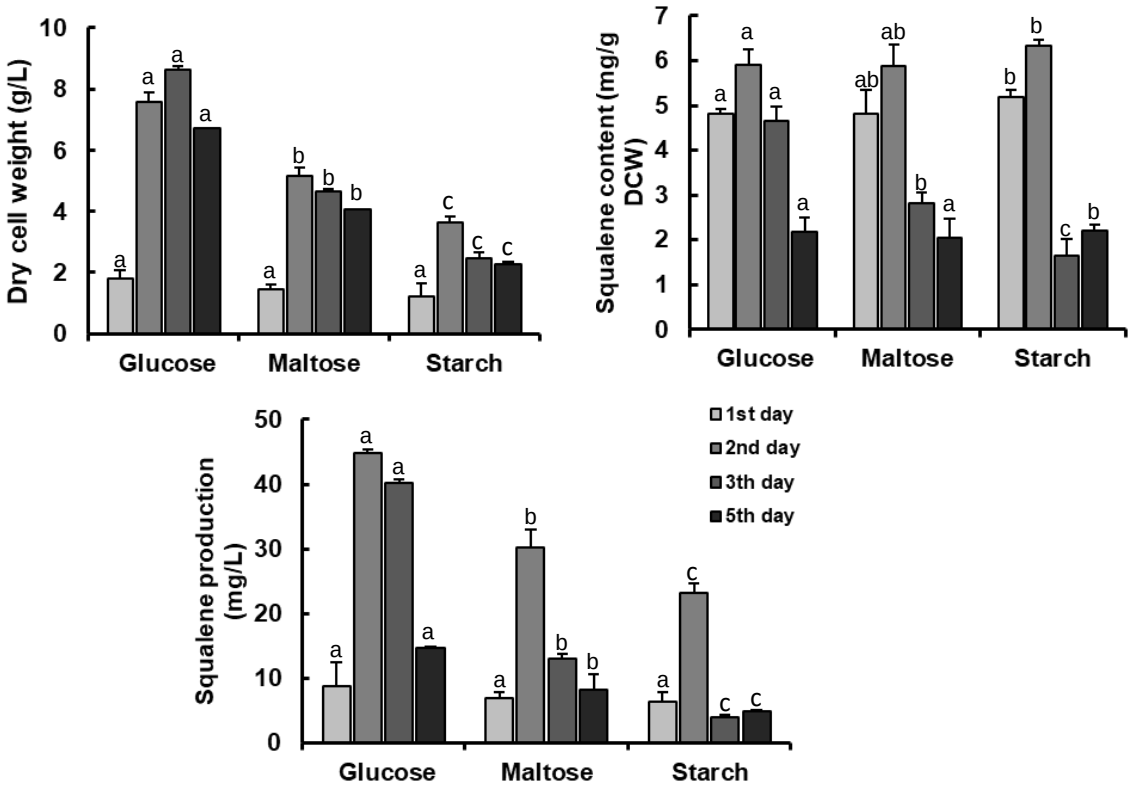

Figure 1. Effect of carbon sources on cell growth and squalene accumulation in *Thraustochytrium* sp. TN22. Data represent the means ± SE (n = 3). a,b,c: Values in a line not sharing a common superscript differ significantly at $P < 0.05$

Glucose concentration test

To obtain a cell growth and squalene accumulation, the effect of glucose concentration was investigated with various concentrations of glucose ranging from 1% to 4%. As shown in Fig. 2, the cell growth of *Thraustochytrium* sp. TN22 increased when glucose concentration was over 1%. The growth rate of TN22 strain achieved the highest value on day 3 in cultivation at all tested glucose concentrations; whereas the maximum level of squalene production occurred on day 2. The medium contained 4% glucose showed the highest biomass, squalene content and production compared to other glucose concentrations. Although, biomass, squalene content and production in the medium contained 2% glucose on day 2 is slightly lower than in the medium contained 4% glucose, the squalene content and production at 2% glucose on day 3 (up to 4.6 mg/g DCW and 38.8 μg/L) were the highest compared to other glucose concentrations. Therefore, further analysis were operated using glucose concentration of 2%.

Our obtained results similar with other groups published as well (Li et al., 2009; Patel et al., 2019). Patel et al. (2019) reported that the squalene contents of *Schizochytrium mangrovei* FB1, *S. mangrovei* FB2, *S. mangrovei* FB3, *S. mangrovei*, *Aurantiochytrium* sp. BR-MP4-A1, *Aurantiochytrium* sp. 18W-13a were of 0.162, 0.08, 0.05, 1.17, 0.72 and 198 mg/g of DCW at glucose concentration of 2% in flask cultivation. Compared to the strains in report of Pora et al. (2014), squalene production of *Thraustochytrium* sp. TN22 was much higher, except for *Aurantiochytrium* sp. 18 W-13a.

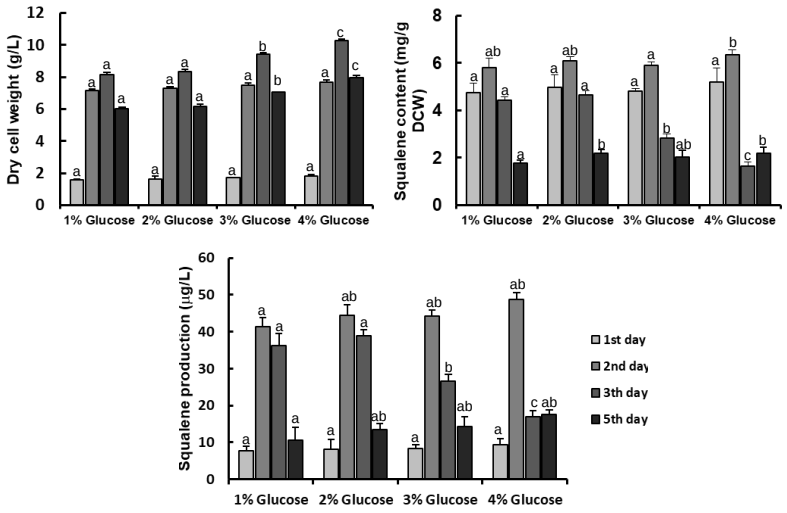

Figure 2. Effect of glucose concentrations on cell growth and squalene production in *Thraustochytrium* sp. TN22. Data represent the means ± SE (n = 3). a, b, c: Values in a line not sharing a common superscript differ significantly at $P < 0.05$

Yeast extract concentration test

The effect of concentrations of yeast extract on cell growth and squalene accumulation of *Thraustochytrium* sp. TN22 were also examined (Fig. 3). The cell growth was increased by increasing the concentration of yeast extract to 1.5%. With all tested yeast extract concentration, the highest dry cell weight of TN22 strain was achieved on day 3, whereas its squalene content reached the maximum on day 2 in cultivation but decreased rapidly thereafter. The highest squalene content (8.9 mg/g of DCW) obtained in the medium containing 0.25% yeast extract but the maximum squalene production was reached 48.9 mg/L in the medium containing 0.5% yeast extract. Thus, 0.5% of yeast extract was selected for subsequent experiment.

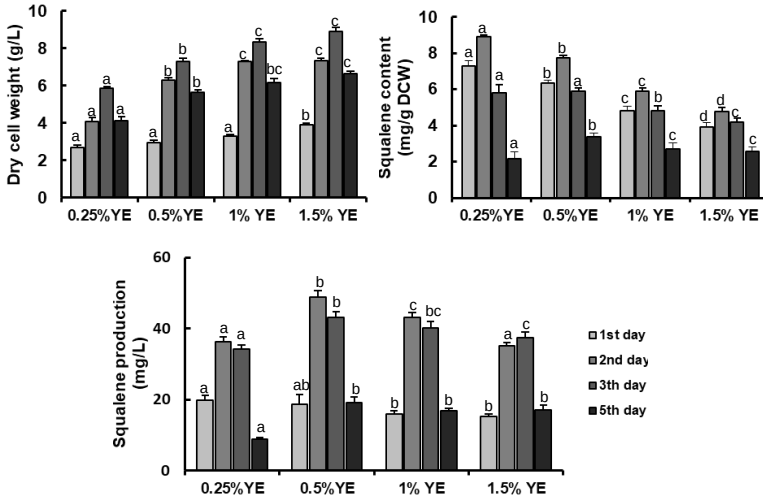

Figure 3. Effect of yeast extract concentrations on cell growth and squalene production in *Thraustochytrium* sp. TN22. Data represent the means ± SE (n = 3). a, b, c, d: Values in a line not sharing a common superscript differ significantly at $P < 0.05$

Vitamin mixture test

Recent studies showed that one of key parameters considering for increasing the production of squalene in species belonging thraustochytrids is the addition of vitamins, more precisely of vitamin B_1, B_6 and especially of vitamin B_{12} (Pora et al., 2014). Among that, vitamin B_{12} would suggest its involvement as a cofactor of some of the key enzymes involved in squalene biosynthesis. Vitamin B_1 would stimulate the leucine degradation pathway, which would increase the intracellular amount of squalene precursors, and vitamin B_6, by modifying the action of cytochromes, would prevent squalene degradation (Pora et al., 2014). Therefore, we investigated the effect of the addition of vitamin mixture on cell growth and squalene production of *Thraustochytrium* sp. TN22 (Fig. 4).

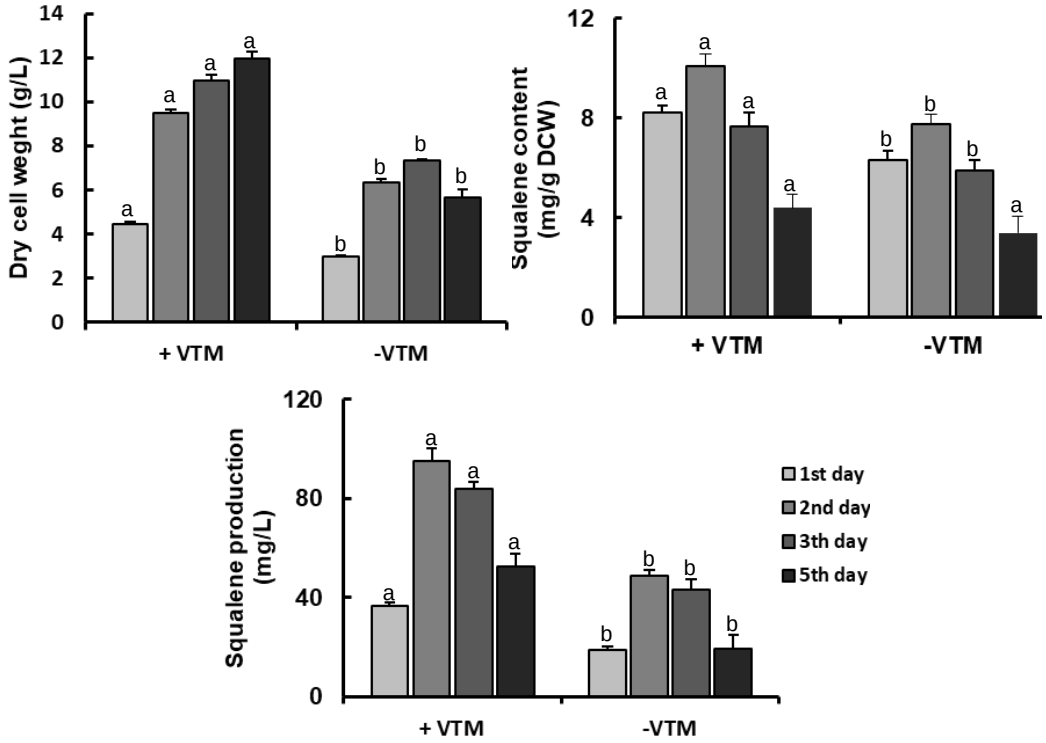

Figure 4. Effect of the addition of vitamin mixture on cell growth and squalene production in *Thraustochytrium* sp. TN22. Data represent the means ± SE (n = 3). a, b: Values in a line not sharing a common superscript differ significantly at $P < 0.05$

The effect of addition of vitamin mixture is remarkable. The DCW and squalene production in the addition of tested vitamin mixture significantly increased 2–3 times higher than that in without adding vitamin mixture. Maximal dry cell weight was observed on day 5 in cultivation, while the highest squalene production was observed on day 2. This trend is in agreement with the report of Pora et al. (2014), Nguyen et al. (2017). Additionally, Nguyen et al. (2017) suggested that the increasing in biomass and squalene production in the medium containing vitamin mixture is due to vitamins enhanced the cells consuming the glucose in the system to increase the growth of cell and the production of squalene.

In comparison with fermented *Schizochytrium* strains in report of Pora et al. (2014) and Nguyen et al. (2017), squalene production of *Thraustochytrium* sp. TN22 was much lower, however, squalene content of the

strain TN22 (9.9 mg/g of DCW) was higher compare those level in *Schizochytrium* sp. ATCC 20888 (4 mg/g of biomass) and *Aurantiochytrium* sp. ATCC PRA 276 (1.2 mg/g of biomass) in the study of Pora et al. (2014). We suggested that the difference compared to the results obtained by Pora et al. (2014), Nguyen et al. (2017) may be due to the different characteristics of the different strains and the difference in culture scale.

Taken together, the highest squalene content and production by *Thraustochytrium* sp. TN22 were 9.9 mg/g DCW and 95.3 mg/mL, respectively with medium containing 2% glucose, 0.5% yeast extract and 0.14% vitamin mixture at 28 ^{o}C after 2 days of cultivation.

Extraction, purification and structural identification of squalene from the cell suspensions of *Thraustochytrium* sp. TN22

In here, we developed a simple and easy method for extraction of squalene from the cell suspensions of *Thraustochytrium* sp. TN22.

One of important factors in squalene extraction process is the rupturing of the cell membranes. According to Pora et al. (2014), cell membrane of thraustochytrids can be lysed easily in medium with pH = 10 at high temprature. Thefore, we first investigated the effect of alkaline medium and time-dependance on the rupturing of the *Thraustocytrium* sp. TN22 cells. The cell suspensions of strain TN22 was adjusted to pH = 10 by KOH 45% and maintained for 1, 2, 4, 6 and 8 hours at 60 ^{o}C, stiring at 150 rpm (Fig. 5). As shown in Fig. 5, the rupturing of cell membrane were in a time dependent manner. The amount of cell lysate was reached by 20% after 1 h, 40–50% after 2 h, 60–70% after 4 hours and almost the cell membranes were broken down after 6 hours. We, therefore, suggested that the condition for completed cell lysis was in 6 hours.

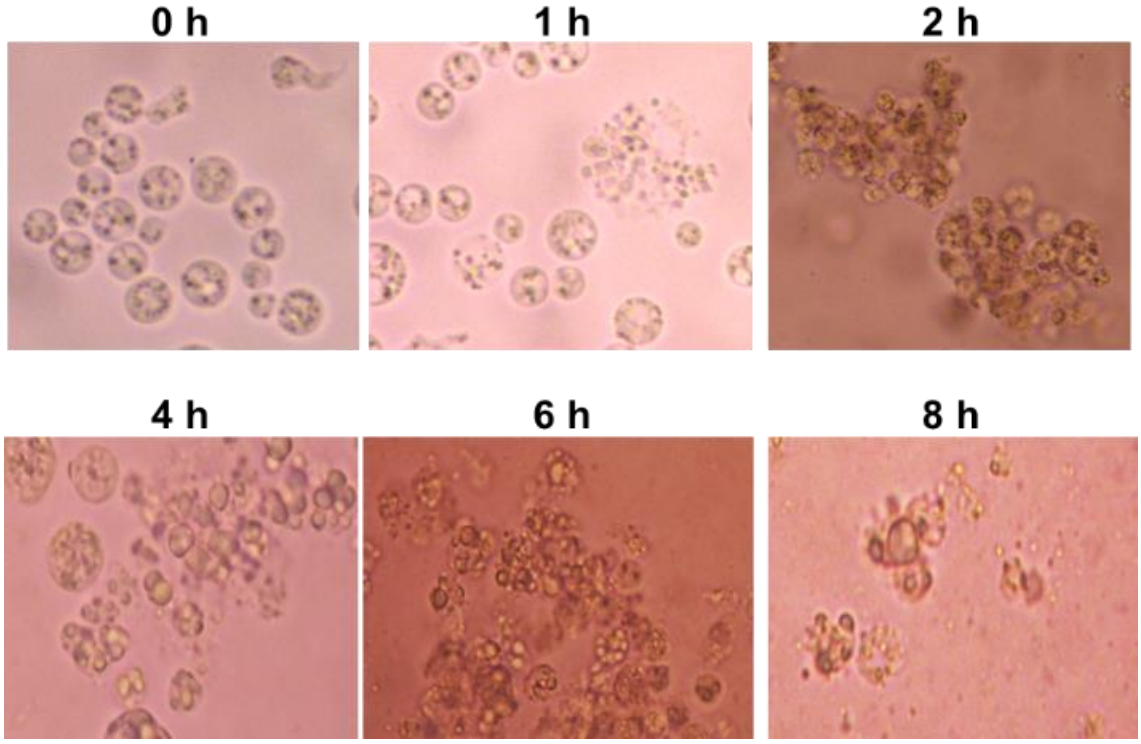

Figure 5. Morphology of *Thraustochytrium* sp. TN22 at the different times in alkaline treatment

Squalene is an component in unsaponifiable lipid. Numerous studies indicated that unsaponifiable lipids from strains of the thraustochytrids can be separated from solvent systems such as n-hexane; methanol: chloroform (2:1, v/v), or petroleum ether (Lu et al., 2003; Hoang et al., 2014; Pora et al., 2014). Therefore, we performed the effect of n-hexane; methanol: chloroform (2:1, v/v), or petroleum ether on squalene extraction of cell lysates of *Thraustochytrium* sp. TN22 (Fig. 6).

As shown in Figure 6, the squalene content was significantly influenced by organic solvents. Squalene production was low in methanol: chloroform (2:1, v/v) (up to 159.9 mg/L), or petroleum ether (135.2 mg/L). In constract, when n-hexane was used, high concentration of squalene was obtained with the value of 178.1 mg/L by HPLC analysis.

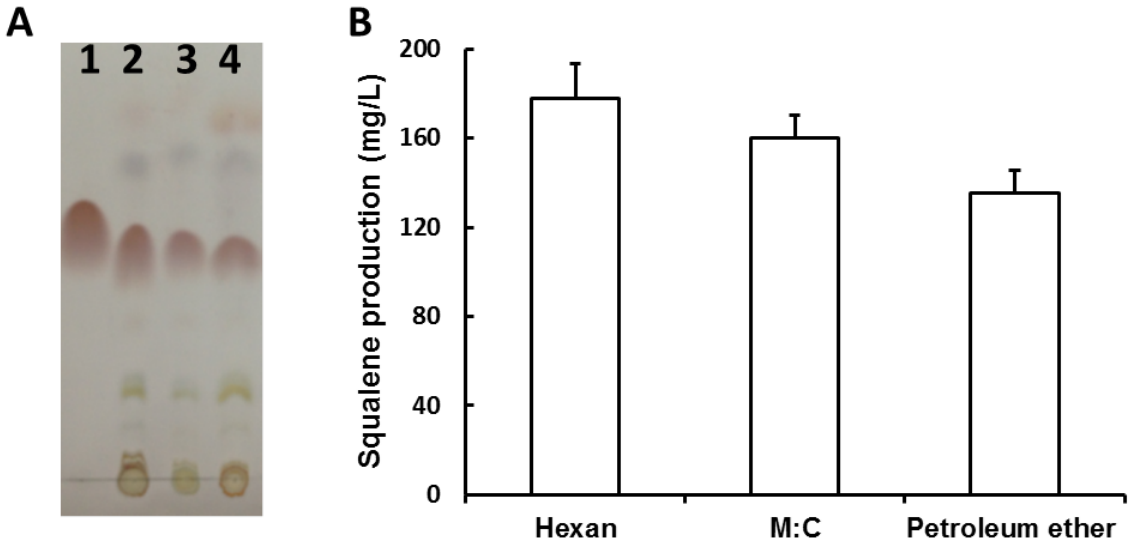

Figure 6. The effect of organic solvent systems on the extraction of squalene from cell lysates of *Thraustochytrium* sp. TN22. A: TLC analysis of extracted squalene from cell lysates where lane 1, squalene standard; lane 2, extrated squalene by n-hexane; lane 3, extrated squalene by methanol: chloroform (M:C, 2:1, v/v); lane 4, extrated squalene by petroleum ether. B: Squalene production. Squalene content was assessed by HPLC analysis

To verify its structure, the squalene obtained after extraction process was purified by silica gel column chromatography. Squalene was sucessfuly separated from n-hexane fraction. Several fractions rich in squalene were detected by TLC with the Sigma standard, combined and then the solvent was evaporated by rotary evaporation to yield a colorless liquid. The squalene in these fractions was then analyzed by HPLC. According to TLC and HPLC data (Fig. 7), extracted squalene was shown to be good purity (90%) with a recovery yield of 70%.

The structure of the squalene from cell suspention was further confirmed by its ^{1}H and ^{13}C NMR spectroscopic data (Fig. 8). The ^{1}H NMR (500 MHz, $CDCl_3$) (Fig. 8A) showed methyl groups at δ 1.60 (s, 18H) and δ 1.68 (s, 6H), methylene groups at δ 1.99–2.02 (m, 20H), and internal vinyl signals at δ 5.08-5.15 (m, 6H). The ^{13}C NMR (125 MHz, $CDCl_3$) (Fig. 8B) showed methyl carbons at δ 16.00, 16.04, 17.67, methylene carbons at δ 25.69, 26.69, 26.80, 28.30, 39.75, 39.77, and double bond carbons at δ 124.30, 124.33, 124.44, 131.22, 134.89, 135.10. The NMR spectra were in

complete agreement with those of the standard squalene material and consistent with the published literature Poucher and Behnke (1993).

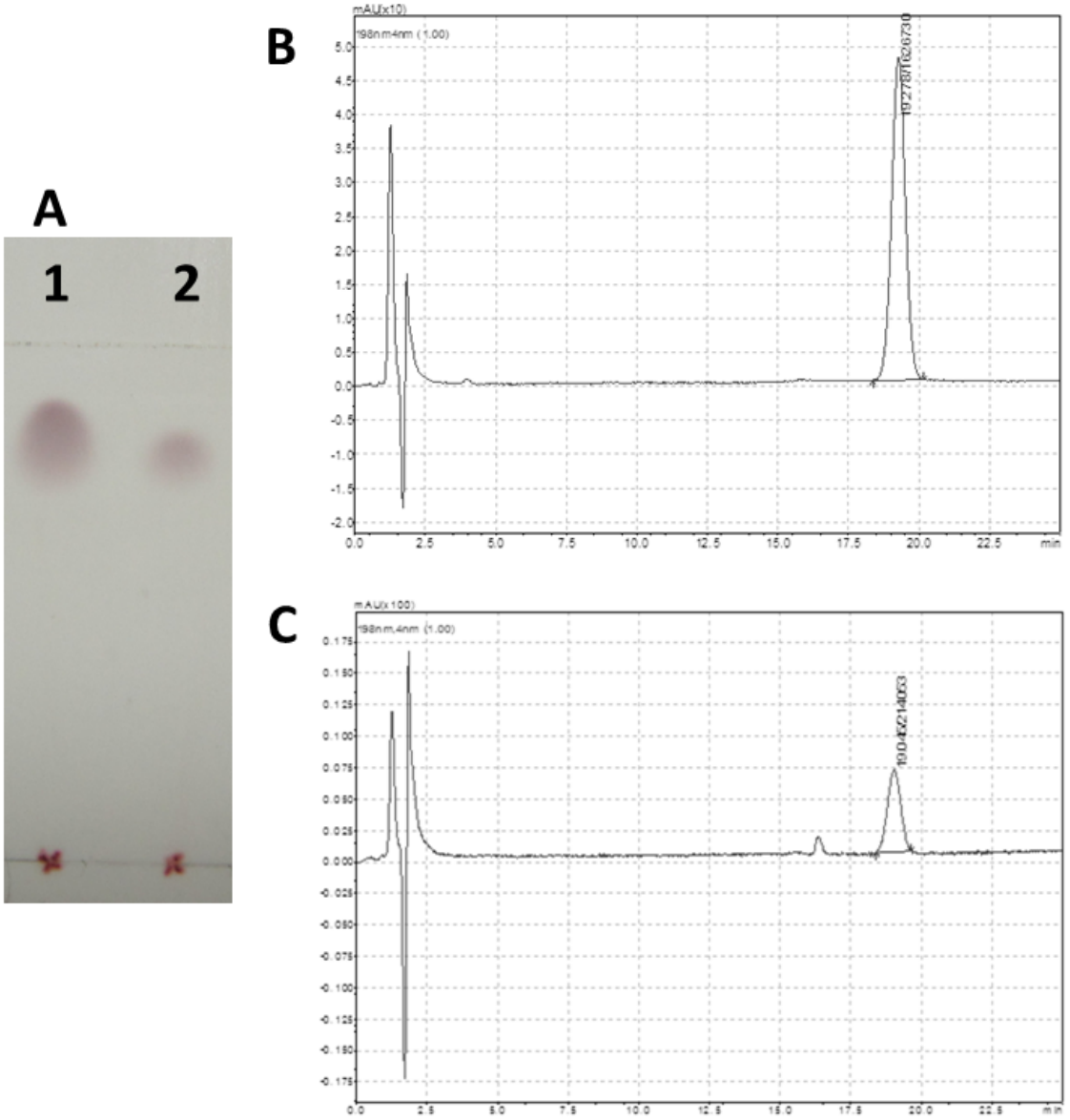

Figure 7. Thin-layer chromatography (A) and typical chromatogram of purified squalene from standard (B) and *Thraustochytrium* sp. TN22 (C). In A: Lane 1, squalene standard; lane 2, squalene purified from cell suspension of *Thraustochytrium* sp. TN22

Taken together, we suggested that the optimal process for extraction of squalene for 100 g biomass/L of cell suspension of *Thraustochytrium* sp. TN22 was cell lysate at alkaline medium (pH = 10), at 60 ^{o}C for 6 h with 1V of each extraction solvents as ethanol and n-hexane. Squalene was then purified on silica gel column 60 using a mobile phase consisting of n-hexane at a flow rate of approximately 1.0 mL/min. This method yielded 178.1 mg squalene at 90% purity from 100 g of biomass.

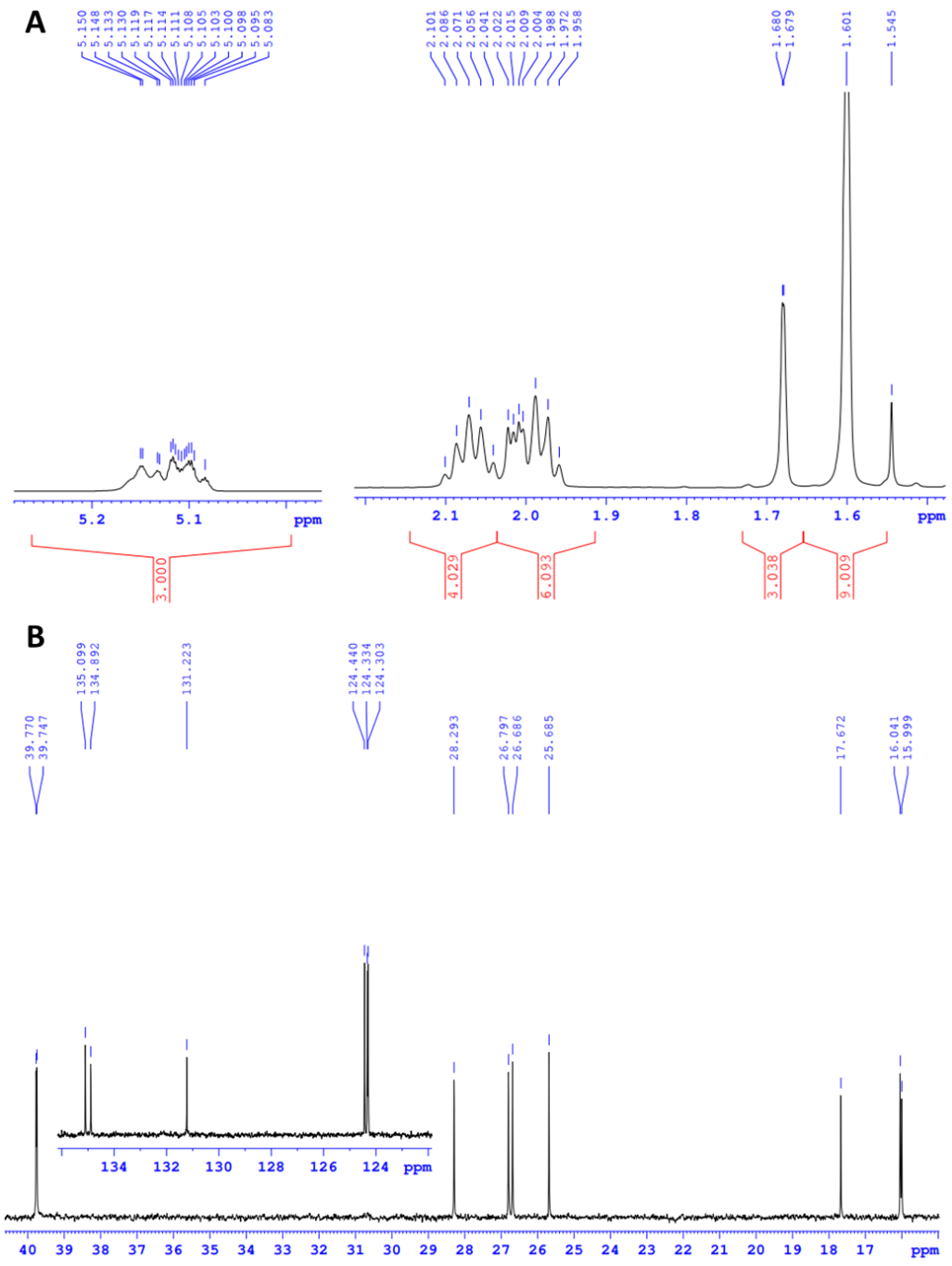

Figure 8. NMR spectra of purified squalene from cell suspension of *Thraustochytrium* sp. TN22. ^{1}H NMR (500 MHz, $CDCl_3$) spectra (A). ^{13}C NMR (125 MHz, $CDCl_3$) spectra (B)

CONCLUSION

In the present study, the cultivation parameters for attaining high squalene accumulation by *Thraustochytrium* sp. TN22 in flasks were optimized. The highest squalene content (9.9 mg/g of DCW) were

obtained with 2% glucose, 0.5% yeast extract and 0,14% mixture of vitamins at 28 ^{o}C in day 2 of cultivation. In addition, the simple method was successful developed to separate and purify squalene 90% purity and 70% revovery of yield.

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