Bioconversion of Glycerol to Docosahexaenoic Acid by Thraustochytrium WB-02 an Indigenous Indonesian Microalga Strain

Witono Basuki
Center for Bioindustrial Technology, Agency for the Assessment and Application of Technology (BPPT), Serpong 15314, Indonesia, witono.basuki@bppt.go.id

Follow this and additional works at: https://scholarhub.ui.ac.id/science

Recommended Citation
Basuki, Witono (2019) "Bioconversion of Glycerol to Docosahexaenoic Acid by Thraustochytrium WB-02 an Indigenous Indonesian Microalga Strain," Makara Journal of Science: Vol. 23 : Iss. 2 , Article 6.
DOI: 10.7454/mss.v23i2.11048
Available at: https://scholarhub.ui.ac.id/science/vol23/iss2/6

This Article is brought to you for free and open access by the Universitas Indonesia at UI Scholars Hub. It has been accepted for inclusion in Makara Journal of Science by an authorized editor of UI Scholars Hub.
Bioconversion of Glycerol to Docosahexaenoic Acid by Thraustochytrium WB-02
an Indigenous Indonesian Microalga Strain

Cover Page Footnote
The author would like to thank Dini Ayudia and Adhi Pradana (the Center for Bioindustrial Technology, Agency for the Assessment and Application of Technology) who assisted me in this study.

This article is available in Makara Journal of Science: https://scholarhub.ui.ac.id/science/vol23/iss2/6
Bioconversion of Glycerol to Docosahexaenoic Acid by *Thraustochytrium* WB-02
an Indigenous Indonesian Microalga Strain

Witono Basuki

Center for Bioindustrial Technology, Agency for the Assessment and Application of Technology (BPPT), Serpong 15314, Indonesia

*E-mail: witono.basuki@bppt.go.id*

Received March 6, 2019 | Accepted June 18, 2019

Abstract

Glycerol is a by-product of the biodiesel industry, and it can be processed to produce many useful derivatives. This study is aimed at examining the bioconversion of glycerol to docosahexaenoic acid (DHA) using local microalgae. Glycerol to docosahexaenoic acid converting microalgae were obtained from the mangrove area in the coastal sea of Lampung Province. The single colony was then generated by the scratching technique in its isolation and purification process. By using 18S rDNA, a potential strain namely WB-02, was identified as *Thraustochytrium* sp. Gas chromatography analysis was performed to identify its product conversion. As a result, *Thraustochytrium* WB-02 was identified to utilize glycerol as a single carbon source and convert to DHA. A maximum DHA yield of more than 3.4 g/L was obtained when the glycerol concentration in the medium was 8%. *Thraustochytrium* WB-02 was regarded as a potential microalgae resource in producing DHA due to its high level of production.

Keywords: bioconversion, docosahexaenoic acid, isolation, glycerol, *Thraustochytrium* WB-02

Introduction

Glycerol is the primary by-product in the biodiesel industry. In general, in the production of 100 kg of biodiesel, 10 kg of glycerol is usually produced [1]. The Ministry of Energy and Mineral Resources reported that in 2016 Indonesia's biodiesel production reached 3.656 billion liters [2], so that the potential of glycerol produced was around 0.366 billion liters.

Several avenues for utilizing glycerol have been investigated. For example, glycerol can be converted to propylene glycol [3] or acetol [4]. It can also be used in the fermentation process to produce 1,3-propanediol [5], lipids [6], pigments [7], and a mixture of succinic acid, butanol, ethanol and hydrogen [8]. Other studies have also shown that glycerol can be used to produce DHA [9].

Studies have shown that docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are important for proper fetal development - including the development neuronal, retinal, and immune function. DHA and EPA may also affect many aspects of cardiovascular function-including inflammation, peripheral artery disease, major coronary events, and anticoagulation. In addition, EPA and DHA have been linked to promising results in prevention, weight management, and cognitive function in those with very mild Alzheimer’s disease [10].

The major commercial source of DHA is fish oil [11]. However, global catches have been in decline since the late 1980s and the number of overfished stocks has been increasing exponentially since the 1950s [12]. Furthermore, the presence of chemical contaminants (such as, mercury) in fish oil can be harmful to consumers. In addition, fish oil is not suitable for vegetarians and the odor makes it unattractive. There are several alternative sources of EPA and DHA - such as bacteria, fungi, plants and microalgae - that are currently being explored for commercial production [13]. The development of microalgae containing DHA from glycerol is an opportunity to provide alternative omega-3 sources. The omega-3 fatty acids can be extracted from algae and used to enrich the nutrition of other foods. Microalgae cell biomass can also be used directly as a feed additive in various animal industries such as aquaculture [14] or poultry [15].

The microalgae belonging to the *Labyrinthulomycetes* class and family *Thraustochytriaceae* [16] are unicellular protists that are present in the marine ecosystem. They play a key role in the initial stage of microalgae chain food, as organic matter degraders. The geographic
distribution of these microorganisms includes Antarctica, the North Sea, India, Micronesia, Japan, and Australia [17]. Recently, the *Thraustochytrium* strain was isolated from the coast of the King George Islands Antarctic [18], Vancouver Island, British Columbia [19], Yundang Lake, Xiamen City, China [20], the coastal waters of Southern China [21], Tasmania and Queensland, Australia, [22], Subic Bay, Philippines [23] and the mangrove areas in Malaysia [24].

However, to the author’s knowledge, there is limited research regarding the isolation of *Thraustochytrium* sp. from Indonesia. This study is aimed at examining the ability to convert glycerol to DHA. These microalgae are expected to be a source for commercial DHA production from glycerol.

**Materials and Methods**

**Materials.** The materials used in this research were glycerol, glucose, yeast extract, peptone, bacteriological agar, streptomycin, penicillin G, polypeptone, methanolic HCl, methylene chloride, icosanoic acid, and n-hexane.

**Sampling.** Sample of fallen leaves were obtained from the mangrove area in Pahawang Islands, Lampung Province, Indonesia. This location was chosen because *Thraustochytrium* spp are monocentric protists and are easily found in marine habitats and mangrove areas. The fallen leaf samples were collected and washed with sterile sea water to remove any particulates, which can be a source of contaminants [25]. The samples were then placed in a container filled with ice and taken to the laboratory in less than four hours to isolate microalgae.

**Isolation of DHA-producing microalgae.** The isolation of DHA-producing microalgae was carried out by the direct plating technique according to the method described by Perveen et al. [26] with a slight modification. The small cut of leaf samples were directly placed on agar plates containing 0.5% (w/v) glucose, 0.1% (w/v) yeast extract, 0.1% (w/v) peptone, 1% (w/v) agar, streptomycin and penicillin G (0.3 g/L each) in 50% (v/v) sea water (referred to as the By+ media) and incubated at 28 °C for several days [25]. The colonies were taken from the plate and re-grown in a plate containing the By+ media. The isolates grown on the By+ media were then purified by the scratching technique until a single colony was obtained. Pure isolates were then inoculated on an agar slant containing the By+ media and incubated at 30 °C for 72 hours and stored at 4 °C as a stock culture. The microorganisms were maintained in slant cultures with a glucose-yeast extract -peptone agar media, containing 2% glucose, 1% polypeptone, 0.5% yeast extract, and 1.5% agar in 50% sea water [27]. The natural seawater in this study was used after filtration.

**Bioconversion of glycerol to DHA.** *Thraustochytrium* WB-02 was inoculated to 10 mL culture media in a 50 mL conical flask. The culture media consisted of 3.0% glycerol and 1.0% yeast extract in 50% (v/v) sea water. The pH of the medium, which was originally around 6, was not adjusted unless otherwise stated. The *Thraustochytrium* WB-02 strain was previously pre-cultured at room temperature (28-30 °C) on a shaker at a speed of 180 rpm for one day. Then about 0.1 mL of the pre-culture broth (5.4 x 10⁶ CFU/mL) was transfer to 10 mL of culture media in 50 mL conical flask to produce an optical density with wavelength of 600 nm (OD600) value of 0.1 or an inoculum size of about 1% (v/v). Fermentation was then carried out on a reciprocal shaker at room temperature (28-30 °C) for 2-3 days under the same conditions. Total lipid content was determined using Bligh and Dyer’s method of extraction [28].

**Microalgae cell growth and lipid analysis.** The microalgae cell growth was determined by dry cell weight. Cells (usually from 2 mL of culture broth) were harvested and washed with water by centrifugation, dried at 105 °C for 3 h and weighed. The fatty acids were directly transmethylated from the dried cells with 10% methanolic HCl and methylene chloride [29]. As an internal standard, 1.0 mg icosanoic acid was usually added to the reaction mixture. The esterified fatty acids were extracted with n-hexane, and the resultant extracts were applied to a gas chromatography analysis.

**Gas chromatography analysis.** Gas chromatography analysis was carried out using the Shimadzu GC-MS QP 2010 equipped with a flame ionization detector with capillary column Rtx-5ms (30 m × 0.25 mm i.d., film thickness 0.25 mm). Helium was used as the carrier gas and the speed was maintained at 0.90 mL/minute and split ratio 100.0. As mentioned above, 1 mL of fatty acid methyl ester was injected with the injection port temperature at 250 °C, oven column temperature at 80 °C, and pressure at 56.9 kPa. The column temperature was maintained at 80 °C for 2 minutes, 210 °C for 1 minutes and increased to 280 °C after 10 minutes. The identification of fatty acid methyl ester components was carried out using the Wiley 7 and NIST 147 library software found in the Shimadzu GC-MS QP 2010. Total fatty acids were calculated from the number of chromatogram peak areas relative to the internal standard peak area. The result of DHA were determined by the composition of DHA multiplied by the total fatty acid content [27].

**Microalgae identification.** Genomic DNA from a pure culture of the best potential, WB-02, was extracted and purified. The DNA was then amplified by a polymerase chain reaction with a 27F primer (5'-GAG TTT GAT CCT GGC TCA G-3') and 1525R primer (5'-AGA AAG GAG GTG ATC CAG CC-3'). The polymerase chain reaction program consisted of pre-denaturation at 96 °C.
for 3 minutes, and then 30 cycles of denaturation at 96 °C for 45 seconds, annealing at 56 °C for 30 seconds, and elongation at 72 °C for 2 minutes. Post elongation was done at 72 °C for seven minutes and finally held at 4°C until the process was completed. The 18S rDNA sequences (500 nt) of WB-02 were then analyzed for similarities using the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/) [30].

The Effect of glycerol concentration on the conversion of glycerol to DHA. The isolate with the best potential Thraustochytrium WB-02 was inoculated to a 10 mL media in a 50 mL flask. The media contained glycerol at various concentrations (0%, 1%, 2%, 3%, 4%, 5%, 8%, 10%, 12%, 15%) and 1.0% yeast extract in 50% (v/v) sea water. The Thraustochytrium WB-02 strains were previously pre-cultured at room temperature (28-30 °C) on a shaker at a speed of 180 rpm for one day, and then about 0.1 mL of pre-culture broth (5.4 x 10^6 CFU/mL) was transferred to 10 mL of culture media in a 50 mL conical flask with an OD_{600} value of 0.1 nm or the inoculum size around 1% (v/v). Fermentation was then carried out using the reciprocal shaker at room temperature (28-30 °C) for 2-3 days. At the end of the fermentation cell growth measurements were carried out on OD_{600}, dry cell weight and the amount of DHA produced by GC-MS chromatographic analysis.

Results and Discussion

Isolation of microalgae. From purified isolates regenerated on the By+ media, 23 isolates showed the ability to produce DHA. After observing the GC-MS chromatography data, WB-02 was chosen as the isolate with the strongest ability to produce DHA from glycerol.

Characterization and identification of isolates. The micrograph of the WB-02 isolate (Figure 1) showed that the WB-02 strain is a spherical, single cell organism. In addition, the 18S rDNA sequence showed that the WB-02 strain has 93% similarity to Thraustochytrium sp. Therefore, the strain WB-02 was named Thraustochytrium WB-02.

Analysis of DHA conversion. Fatty acid chromatograms as a result of the conversion of glycerol to fatty acids are shown in Figure 2, while fatty acid components are shown in Table 1. From Table 1 it can be observed that by using glycerol as a carbon source, Thraustochytrium WB-02 can produce several polyunsaturated fatty acids, namely: 5,8,11,14-eicosatetraenoic acid (0.42%), 5,8,11,14,17-eicosapentaenoic acid (0.84%) and 4,7,10,13,16,19-docosahexaenoic acid (44.88%). It appears that DHA is the main component with a portion reaching almost 45%. Thus, it can be said that Thraustochytrium WB-02 is a potential microbial converting glycerol to DHA.

Figure 1. Micrograph of Thraustochytrium WB-02

Figure 2. Nyquist Plot of the Double-Layer Composite of TiO2/CNT/AC at A 0.1–100,000 Hz Frequency Range. The Mass Fractions are (a) Sample A, (b) Sample B, (c) Sample C, and (d) Sample D
Table 1. Component of Fatty Acids Resulting from the Bioconversion of *Thraustochytrium* WB-02

| No. | Peak | Retention Time (min) | Percentages Area (%) | Components                                      |
|-----|------|----------------------|----------------------|-------------------------------------------------|
| 1   | 1    | 10.070               | 0.15                 | Dodecanoic acid, methyl ester                    |
| 2   | 2    | 10.871               | 0.58                 | 2,4,6-tri-tert-butylphenol                       |
| 3   | 3    | 12.417               | 9.90                 | Tetradecanoic acid, methyl ester                 |
| 4   | 4    | 13.500               | 12.67                | Pentadecanoic acid, methyl ester                 |
| 5   | 5    | 14.525               | 23.77                | Hexadecanoic acid, methyl ester                  |
| 6   | 6    | 14.852               | 1.13                 | Heptadecanoic acid                               |
| 7   | 7    | 15.567               | 2.99                 | Heptadecanoic acid, methyl ester                 |
| 8   | 8    | 16.775               | 2.29                 | Octadecanoic acid, methyl ester                  |
| 9   | 9    | 18.355               | 0.42                 | 5,8,11,14-eicosatetraenoic acid, methyl ester    |
| 10  | 10   | 18.442               | 0.84                 | 5,8,11,14,17-eicosapentaenoic acid, methyl ester |
| 11  | 11   | 20.800               | 44.88                | 4,7,10,13,16,19-docosahexaenoic acid, methyl ester|
| 12  | 12   | 26.621               | 0.39                 | Tricosanoic acid, methyl ester                   |

100.0

Figure 2. Nyquist Plot of the Double-Layer Composite of TiO2/CNT/AC at A 0.1–100,000 Hz Frequency Range. The Mass Fractions are (a) Sample A, (b) Sample B, (c) Sample C, and (d) Sample D

Effect of Glycerol Concentration on *Thraustochytrium* WB-02 Growth. *Thraustochytrium* WB-02 - which is an indigenous Indonesian microalga strain isolated from Lampung Province, Indonesia-has the potential to convert glycerol to DHA. Figure 3 shows the effect of glycerol concentration on *Thraustochytrium* WB-02 growth and the DHA produced. The experiment showed the highest *Thraustochytrium* WB-2 growth when the glycerol concentration was 8%, with the highest concentration of DHA produced as 3.4 g/L. In Chi et al.’s study [31] optimizing the conditions of the culture resulted in the production of 4.91 g/L of DHA. Several attempts have been made to increase the production of DHA produced by *Thraustochytrium*. By changing the carbon and nitrogen sources in the culture media of the *Schizochytrium* sp, Sahin et al. [32] could affect the production of biomass, fatty acids and DHA. The highest biomass and yield were obtained by using
peptone proteose as the only nitrogen source. The combination of peptone proteose as a source of nitrogen and glycerol as a carbon source, as well as the addition of ethanol with the selection of the right time proved to be beneficial for obtaining higher DHA yields [32]. Furthermore, by optimizing the use of response surface methodology, Manikan et al. [33], could increase the amount of DHA produced by *Thraustochytrium* from Lampung Province, Indonesia - has the potential to convert glycerol to DHA. Its productivity in producing DHA can, however, still be improved by optimizing the conditions of cultivation and media composition during glycerol fermentation to DHA.

**Conclusion**

It can be concluded that *Thraustochytrium* WB-02 - which is an indigenous Indonesian microalga strain isolated from Lampung Province, Indonesia - has the potential to convert glycerol to DHA. Its productivity in producing DHA can, however, still be improved by optimizing the conditions of cultivation and media composition during fermentation.

**Acknowledgments**

The author would like to thank Dini Ayudia and Adhi Pradana (the Center for Bioindustrial Technology, Agency for the Assessment and Application of Technology) who assisted me in this study.

**References**

[1] Van Gerpen, J., Shank, B., Pruszko, R., Clements, D., Knothe, G. 2004. Biodiesel Production Technology, August 2002- January 2004. Subcontractor Report. National Renewable Energy Laboratory, Colorado USA. pp.1-6.

[2] USDA. 2017. Indonesia Biofuel Annual Report 2017. GAIN Report ID 1714. Foreign Agricultural Service, Jakarta.

[3] Tanielyan, S.K., Marin, N., Alvez, G., Bhagat, R., Miryala, B., Augustine, R.L., Schmidt, S.R. 2014. An efficient, selective process for the conversion of glycerol to propylene glycol using fixed bed Raney copper catalysts. Org. Process. Res. Dev. 18(11): 1419-1426, https://doi.org/10.1021/op400123f.

[4] Braga, T.P., Essayem, N., Prakash, S., Valentini, A. 2016. Gas-phase conversion of glycerol to acetal: Influence of support acidity on the catalytic stability and copper surface properties on the activity. J. Braz. Chem. Soc. 27(12): 2361-371, https://dx.doi.org/10.5935/0103-5053.20160134.

[5] Przystalowska, H., Lipiński, D., Slomski, R. 2015. Biotechnological conversion of glycerol from biofuels to 1,3- propanediol using Escherichia coli. Acta Biochim. Pol. 62(1): 23-34. http://dx.doi.org /10.18388/abp.2014_885.

[6] Canonico, L., Ashoor, S., Taccari, F., Antonucci, M., Truzzi, C., Scarpone, G., Ciani, M. 2016. Conversion of raw glycerol to microbial lipids by new Metschnikowia and Yarrowia lipolytica strains. Ann. Microbiol. 66(4): 1409–1418, https://doi.org/10.1007/s13213-016-1228-0.

[7] Tallapragada P., Dikshit, R., Dessai, P.T. 2013. Effect of glycerol as a sole carbon source on Monascus sp. for pigment production. Int. Food. Res. J. 20(6): 3265-3268, http://www.ifrj.upm.ed u.my.

[8] Pradima, J., Kulkarni, M.R. Archna, 2017. Review on enzymatic synthesis of value added products of glycerol, a byproduct derived from biodiesel production. Resource-Efficient Technol. 3(4): 394-405, https://doi.org/10.1016/j.refit.2017.02.009.

[9] Lung, Y.T., Tan, C.H., Show, P.L., Ling, T.C., Lan, J.C.W., Lam, H.L., Chang, J.S. 2016. Docosahexaenoic acid production from crude glycerol by Schyzochitrium limacinum SR21. Clean Technol. Environ. Pol.18(7): 2209-2216, https://doi.org/10.1017/s10098-016-1126-y

[10] Swanson, D., Block, R., Mousa, S.A. 2012. Omega-3 fatty acids EPA and DHA: health benefits throughout life. Adv. Nutr. 3(1): 1-7, https://doi.org/10.3945/an.111.000893.

[11] Ganesan, B., Brothersen, C., McMahon D.J. 2014. Fortification of foods with omega-3 polyunsaturated fatty acids. Crit. Rev. Food. Sci. Nutr. 54(1): 98-114, http://dx.doi.org/10.1080/10408398. 2011.578 221.

[12] Food and Agriculture Organization, 2018. The State of World Fisheries and Agriculture. Meeting the Sustainable Development Goals. Food and Agriculture Organization of the United Nations, Rome. pp. 2-7.

[13] Adarme-Vega, T.C., Lim, D.K.Y., Timmins, M., Vernen, F., Li, Y., Schenk, P.M. 2012. Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production. Microb. Cell. Fact. 11: 96-105, http://doi.org/10.1186/1475-2859- 11-96.

[14] Lee, M.C., Zhuo, L.C., Lin, Y.H. 2018. Effects of dietary docosahexaenoic acid sources, microalgae meal and oil, on growth, fatty acid composition and docosahexaenoic acid retention of orange-spotted grouper, Epinephelus coioides. Aquaculture Res. 49(1): 30-35, http://doi.org/10.1111/aer.13429.
[15] Cherian, G. 2015. Nutrition and metabolism in poultry: role of lipids in early diet. J. Anim. Sci. Biotechnol. 6(1): 28, doi: 10.1186/s40104-015-029-9.
[16] Raghukumar, S. 2002. Ecology of the marine protists, the Labyrinthulomyxetes (Thraustochytrids and Labyrinthulids). Europ. J. Protistol. 38(2), 127–145, http://dx.doi.org/10.1016/S0967-0468(02)00118-3.
[17] Lewis, T.E., Nichols, P.D., McMeekin, T.A. 1999. The Biotechnology Potential of Thraustochytrids. Mar. Biotechnol. 1(6): 580-587, https://doi.org/10.1007/PL00011813.
[18] Caamano, E., Loperena, L., Hinzpeter, I., Pradel, P, Gordillo, F., Corsini, G., Tello, M., Lavin, P. González, A.R., 2017. Isolation and molecular characterization of Thraustochytrium strain isolated from Antarctica Peninsula and its biotechnological potential in the production of fatty acids. Braz. J. Microbiol. 48(4): 671-679, doi: 10.1016/j.bjm.2017.01.011.
[19] FioRito, R., Leander, C., Leander, B. 2016. Characterization of three novel species of Labyrinthulomyxota isolated from ochre sea stars (Pisaster ochraceus). Mar. Biol. 163:170, https://doi.org/10.1007/s00227-016-2944-5.
[20] Wang, Z., Lou, S., Hu, F., Wu, P., Yang, L., Li, H., He, L., Lin, X. 2016. Complete mitochondrial genome of a DHA-rich protist Schizochytrium sp. TIO1101. Mitochondrial DNA Part B. 1(1): 126-127, https://doi.org/10.1080/23802359.2016.1144490.
[21] Liu, Y., Singh, P., Sun, Y., Luan, S, Wang, G. 2014. Culturable diversity and biochemical features of thraustochytrids from coastal water of Southern China. Appl. Microbiol. Biotechnol. 98(7): 3241-3255, doi:10.1007/s00253-013-5391-y.
[22] Lee-Chang, K.J., Dunstan, G.A., Abell, G.C., Clementson, L.A., Blackburn, S.I., Nichols, P.D., Koutoulis, A. 2012. Biodiversity of new Australian thraustochytrids for production of biodiesel and long-chain omega-3 oils. Appl. Microbiol. Biotechnol. 93(5): 2215-2231, https://doi.org/10.1007/s00218-011-2515-4.
[23] Arafiles, K.H.V., Alcantara, J.C.O., Cordero, P.R.F., Batoon, J.A.L., Galura, F.S., Leaño, E.M., Dedeles, G.R. 2011. Cultural optimization of Thraustochytrids for biomass and fatty acid production. Mycosphere. 2(5): 521–531.
[24] Hong, W.K., Rairakhwada, D., Seo, P.S., Park, S.Y., Hur, B.K., Kim, C.H., Seo, J.W. 2011. Production of lipids containing high levels of docosahexaenoic acid by a newly isolated microalgae, Auratiomyxoma sp. KRS101. Appl. Biochem. Biotechnol. 164(8): 1468-1480, https://doi.org/10.1007/s12010-011-9227-x.
[25] Gupta, A., Barrow, C.J., Puri, M. 2012. Omega-3 biotechnology: Thraustochytrids as a novel source of omega-3 oils. Biotechnol. Adv. 30(6): 1733-1745, https://doi.org/10.1016/j.biotechadv.2012.02.014.
[26] Perveen, Z., Ando, H., Ueno, A., Ito, Y., Yamamoto, Y., Yamada, Y., Takagi, T., Kaneko, T., Kogame, K., Okuyama, H. 2006. Isolation and characterization of a novel thraustochytrid-like microorganism that efficiently produces docosahexaenoic acid. Biotechnol. Lett. 28(3): 197-202, https://doi.org/10.1007/s10529-005-5335-4.
[27] Yokochi, T., Honda, D., Higashihara, T. Nagahara, T., 1998. Optimization of docosahexaenoic acid production by Schizochytrium limacium SR21. Appl. Microbiol. Biotechnol. 49(1): 72-76, https://doi.org/10.1007/s002530051139.
[28] Bligh, E.G., Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem Physiol. 37(8): 911–917, https://doi.org/10.1139/o59-099.
[29] Shimizu, S., Kawashima, H., Shinmen, Y., Akimoto, K., Yamada, H. 1988. Production of eicosapentaenoic acid by Mortierella fungi. J. Am. Oil. Chem. Soc. 65(9): 1455-1459, https://doi.org/10.1007/BF02898307.
[30] http:// www. ncbi.nlm.nih. Gov / blast /.
[31] Chi, Z., Pyle, D., Wen, Z., Frear, C., Chen, S. 2007. A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation. Process. Biochem. 42(11): 1537-1545, https://doi.org/10.1016/j.procbio.2007.08.008.
[32] Sahin, D., Tas, E., Altiman, U.H. 2018. Enhancement of docosahexaenoic acid (DHA) production from Schizochytrium sp. S31 using different growth medium conditions. AMB. Exp. 8(1):7, https://doi.org/10.1186/s13568-018-0540-4.
[33] Manikan, V., Kaili, M.S., Hamid, A.A. 2015. Response surface optimization of culture medium for enhanced docosahexaenoic acid production by a Malaysian thraustochytrid. Sci. Rep. 5: 8611, https://doi.org/10.1038/srep08611.
[34] Prabu, R., Raksha, S., Karuppuchamy, S. 2012. Effect of sodium sulphate salinity for production of docosahexaenoic acid (DHA) by Thraustochytrids Aurea RAK-21. Asian Biomed. 6(5): 693-701, https://doi.org/10.5372/1905-7415.0605.109.