Tropical marine *Navicula salinicola* NBO: morphology, genetic identification, and biochemical properties

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Abstract. Indonesia's oceans are rich in microalgae as unicellular photosynthetic organisms. So far, most of Indonesia's diverse microalgae have yet been explored. An Isolate NBO of microalgae has been collected from Bokor Island in the Jakarta Bay, Indonesia. This study aimed to identify the isolate NBO and to characterize its biochemical properties. The identification was carried out based on the physical morphology of cell and the nucleotide sequence of 18S rDNA-V4 and LSU D2/D3 DNA regions, meanwhile, the biochemical properties were characterized based on the content of carbohydrate, lipid and fatty acid of cells. The results showed that the morphological characteristics of NBO cells possessing a color of pale yellow, an oval shape ranging from 11.00–15.00 μm in length and 4.67–5.00 μm in width, and a silica frustule that bilaterally symmetrical oval closed to genus *Navicula*. Based on the homology of sequences 402-bp of 18S rDNA-V4 and 591-bp of LSU D2/D3, the isolate was identified as *Navicula salinicola*. Total carbohydrate and lipid contents of *Navicula salinicola* NBO were 13.13 and 3.74% (w/w), respectively. Fatty acids of *Navicula salinicola* NBO were dominated by palmitic acid (C16:0), palmitoleic acid (C16:1), and eicosapentaenoic acid (EPA; C20:5) with a total concentration of 38.24, 37.23, and 12.54% (w/w, fatty acid/lipids), respectively, indicating a promising candidate for biodiesel and nutrition/pharmaceutical.

1. Introduction

Microalgae are unicellular photosynthetic organisms living either as solitary cells or in colonies in either oceans or even water bodies. They are responsible for increasing the biomass in such water bodies owing to their higher rates of cell division. They vary in shape, size, color, and structure. A diatom is a group of microalgae that have a silica (hydrated silicon dioxide) cell wall named frustule [1]. Diatoms have the capacity to produce different biologically active metabolites such as proteins, lipids, and carbohydrates depending on their different genetics, physiology, and phenotype. The quality and quantity of these metabolites are specific to each species, for example, some can accumulate high lipid content especially under stressed conditions. Diatoms have great potential for human life by providing alternatives like foods, biodiesel, medicine [2]. Since a few reports on tropical marine diatoms, this is interesting to explore valuable compounds of them.

We have screened an individual microalgal sample (NBO) obtained from Bokor Island, Jakarta Bay, Indonesia. A common method to identify microalgal strain is by characterization of its cell morphology and/or its genetic profiling. Cell morphological identification is based on the physical
features of a cell such as its shape, color, and size, usually using a light microscope and SEM (Scanning Electron Microscopy). Meanwhile, genetic profiling is based on the specific nucleotide sequence as a marker such as 18S rDNA (gene encoding small subunit (40S) of eukaryotic ribosomes), LSU (gene encoding large subunits of eukaryotic ribosomes), rbcL (gene encoding Ribulose-1,5-bisphosphate carboxylase/oxygenase), or COI (gene encoding Cytochrome c oxidase I). Typical marker genes to identify diatoms are 18S rDNA-V4 (highly variable region 4 of 18S rDNA) and LSU D2/D3 (highly variable domain D2 and D3 of LSU) [3]. In this paper, we identified isolate NBO based on cell physical morphology and genetic profiling of 18S rDNA-V4 and LSU D2/D3 genes. Further biochemical characteristics such as carbohydrate and lipids content of isolate NBO are also reported.

2. Method

2.1. Maintenance and cultivation of isolate NBO
An isolate of NBO was sampled from Bokor Island, Jakarta Bay, Indonesia (S 05° 56.927; E 106° 37.723) on September 14th, 2015 at 10:00 to 14:00 WIB. A single NBO cell was grown in f/2 medium [4], with growth conditions of salinity 27–28 ppt, pH 8.2–8.7, temperature 21–22 °C, light intensity 20.24 µmol cm⁻² s⁻¹, and light and dark period 12:12. To obtain biomass, the cells were grown for 8d. To maintain culture as a laboratory collection, NBO cells were regenerated in a new growth medium every 8 days.

2.2. Identification of morphology and growth of NBO cell
Morphology of NBO cells in the log phase was observed under a light microscope at 400X magnification, and cell sizes were measured under an Optilab camera connected to the computer and the Optilab Viewer and Image Raster 3 program. Before SEM photography, NBO cells were treated by glacial acetic acid (1:1), heated at 60 °C for 30 min, centrifuged at 3500 rpm for 15 min (Thermoscientific SL16R), washed 3 times with deionized water, homogenized in 1 mL of ethanol, and dried overnight in the oven. Dried cells were photographed using SEM (JEOL-JSM-6510LA). The growth rate of NBO cells was analyzed by counting cell numbers daily using a haemocytometer and the cell growth curve was made by plotting the average of cell number to time of growth.

2.3. Isolation of NBO total DNA and Amplification of 18S rDNA-V4 and LSU D2/D3 fragments
NBO total DNA was isolated by Qiagen® Dneasy Plant Mini Kit (Qiagen Inc.). Amplification of 18S rDNA-V4 and LSU D2/D3 was proceeded by PCR using GoTaq Green Master Mix 2X Kit (Promega). Primers to amplify 18S rDNA-V4 were M13F-D512 for 18S (5’-TGTAAAACGACGGCCAGT CAGCTCCAATAGCG-3’) as the forward primer and M13R-D978rev 18S (5’-CAGGAAACGC TATGACGACTACGATGGTGATCTAATC-3’) as the reverse primer, with the PCR profile of initial denaturation at 94 °C for 40 s, 34 cycles of 94 °C for 40 s, 51 °C for 40 s, 72 °C for 1 min, and a final extension of 72 °C for 10 min. PCR amplification of the LSU D2/D3 gene fragment was performed using T16N (5’-AMAAGTACCRYGAGGGAAAG-3’) forward and T24U (5’-SCWCTAATCA TTCGCTTACC-3’) reverse primers. The PCR profile for LSU D2/D3 was initial denaturation at 94 °C for 5 min, 38 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 7 min [3]. NBO total DNA and amplified products of 18S rDNA-V4 and LSU D2/D3 fragments were visualized by electrophoresis in 1.0% agarose gel containing ethidium bromide [5].

2.4. Sequencing of NBO 18S rDNA-V4 and LSU D2/D3 fragments
Sequencing was performed to determine the exact order of the four nucleotide bases (adenine, A; guanine, G; cytosine, C; thymine, T) that make up the DNA molecule. Sequencing reactions of gene fragments of 18S rDNA-V4 and LSU D2/D3 of NBO followed the Sanger Method [6]. The primer used for sequencing of 18S rDNA-V4 fragment was a single primer of M13F (−21) (5’-TGTAAAACGACGGCCAGT-3’) [7], while for LSU D2/D3 fragment was the same forward and reverse primers as described for PCR (Method 2.3). Nucleotide Sequences were analyzed using
several software such as DNA Baser Assembler V4 software [8], BLAST (Basic Local Alignment Search Tool) from NCBI (National Center for Biotechnology Information) [9], Bioedit [10], and MEGA 7 [11]. Nucleotide Sequences of the NBO 18S rDNA-V4 and LSU D2/D3 were submitted on GenBank (NCBI).

2.5. Determination of NBO total carbohydrate
Wet cell biomass harvested from the NBO culture towards their stationary phase was freeze-dried under vacuum for 24h at −40 °C. Total carbohydrate content was determined following the phenol-sulphuric acid method by Dubois et al. (1956) [12]. In this method, cell carbohydrates were digested by sulphuric acid, and the amount of carbohydrates was analyzed based on spectrophotometric methods by measuring the intensity of colors produced from the reaction of glucose with phenol. The color intensity corresponds to the carbohydrate quantity.

2.6. Determination of NBO total lipids and fatty acid profiles
The lyophilized NBO cell biomass of 2.75g was dissolved in chloroform:methanol (2:1), lysed by sonication for 10 min at 40% amplitude, centrifuged at 4000 rpm for 15 min (Thermoscientific SL16R), and the supernatant was collected in an Erlenmeyer flask. The supernatant was added with chloroform and sodium chloride solution, mixed thoroughly, centrifuged at 2000 rpm for 10 min, and the upper layer was transferred to a vial and placed in a rotary evaporator to remove the solvent and to precipitate lipids. Fatty acid methyl esters (FAME) were produced by esterification of the dried lipids. The procedure of FAME production followed the method described by Ehimen et al. (2009) [13]. The fatty acid profiles were determined by gas chromatography equipped with a flame ionization detector (GC–FID). To confirm the FAME components, a set of FAME standards containing 37 different components was also injected into the GC–FID under the same conditions [14].

3. Result and Discussion
An algal NBO sample originating from Bokor Island in the Jakarta Bay, Indonesia, was investigated based on its cell morphology and genetics. Figure 1 shows the images of NBO cells under a light microscope and SEM. NBO cells occurring as single uniform cells were bilaterally symmetrical oval-shaped. The cells were pale yellow-colored with a size ranging from 11.00–15.00μm in length and 4.67–5.00μm in width (Figure 1A). This oval shape could indicate that the samples belong to the diatom group of microalgae.

Figure 1. A NBO cell under light microscope (A) and under SEM (B). A NBO frustule shows two linear-lanceolate valves with rounded ends (1), two raphe (2), a central valve (3), and parallel lineolate striae (4) composed by a row of lineolae (5).
Diatoms are the only organism on this planet whose cell walls are composed of transparent opaline silica with such intricate and striking patterns. The silica net that frames the diatom cell wall is called frustule, which can be observed using SEM. Frustule is formed by two opposite valves and their linking girdle bands. A diatom valve is the siliceous unit at one side of the frustule. Taxonomy of diatoms is mostly based on morphology of the valve that builds the frustule [15]. The NBO frustule was found to have two linear-lanceolate valves with both rounded edges, as shown in Figure 1B. Lanceolate refers to a valve having an elongated outline, widest at the middle, and tapering to both ends [16]. According to Spaulding and Edlund (2008), the valves of Navicula tend to be elliptic to broadly-lanceolate in outline. The valve ends may be capitate, acute, rounded, or not expanded [17]. Based on its valves shape, NBO belongs to the diatom of the genus Navicula. Figure 1B further shows the raphe system of NBO which is composed of two slits, or fissures, that penetrate the valve. Each slit is called a branch of the raphe. The branches are separated by a silica thickening called the central nodule. The SEM image shows the two branches of the raphe system on a single valve of NBO (Figure 1B.2). The raphe system allows the cells to move over surfaces [15]. To assist the movement or mobility, Navicula cells continually secrete a mucilaginous material, which is a form of extracellular polymeric substance (EPS), along the slits [18]. Moreover, NBO has striae, i.e. rows of pores that are extended from the apical axis of raphe to the cell margin (Figure 1B). The pores are called areolae, which could differ in size, shape, or pattern among the diatom species. The size or radius of areolae affects both the diffusion and advection of microscopic and sub-microscopic particles through the diatom cell membrane. Depending on the size of the particle and the radius of the areolae, the cell surface may be capable of preselecting material (macromolecules to bacteria) that reaches the cell membrane and its receptors [19]. The SEM image also shows that NBO areolae took form of small lines elongated in the apical direction. Areolae in form of small lines are called lineolae. Thus, the NBO striae are also called the lineolate striae (Figure 1B). The typical morphology of the NBO sample shows the characteristics of genus Navicula, hence named as Navicula NBO.

To understand growth of Navicula NBO, the cell was cultured in f/2 medium at room temperature. Figure 2 shows the change in cell density daily. Under laboratory conditions, cells of Navicula NBO with an initial density of 50,000 cells/mL needed 5d to adapt to the medium. Exponential cell growth was observed starting from 5th to 8thd with a maximum density of 13,520,000 cells/mL. Entering a stationary phase, at 8thd, Navicula began to produce and store a lot of cellular lipids for surviving in a nutrient-poor environment [20] [21]. After the 9thd, the cell number decreased drastically (the death phase). To produce lipids, Navicula NBO cells were harvested after the 8thd of cultivation, i.e. at the beginning of the stationary phase, because an accumulation of algal oils (mainly triacylglycerols (TAGs)) inside lipid bodies occurred during the stationary phase when the cell growth decreased [21] [22]. The growth curve of Navicula NBO looks similar to that of Navicula sp. cultivated on Walne medium by Nurachman et al. (2012), i.e. the log growth phase (exponential increase in cell number) occurs for three days, and the stationary phase takes only one day until the cells then enter the death phase (decrease of cell number). Meanwhile, the lag phases are found to be different, i.e. it lasts 3 days for the culture of Nurachman et.al instead of 5 days for Navicula NBO. It seems that Navicula grown in f/2 medium requires a longer adaptation time than in the Walne medium [21].
To further identify the species, gene fragments of 18S rDNA-V4 and LSU D2/D3 of Navicula NBO were analyzed. PCR primers used to amplify 18S rDNA-V4 diatom were designed by Zimmermann et al. (2011), while LSU D2/D3 primers were designed or modified by Hamsher et al. (2011) [3]. About 500-bp of the 18S rDNA-V4 gene fragment (Figure 3A) and 600-bp of the LSU D2/D3 fragment (Figure 3B) were obtained and sequenced. A 402-bp length of 18S rDNA-V4 Navicula NBO was obtained and deposited at GenBank with the accession number of MH125175.1 (Figure 4). The sequence of 18S rDNA-V4 of Navicula NBO had 99% identity with that of *Navicula salinicola* (Genbank No. GQ219689.1). This indicates Navicula NBO was closely related to *Navicula salinicola*. Meanwhile, a 591-bp length of LSU D2/D3 of Navicula NBO was also deposited at GenBank with the accession number of MH125176.1 (Figure 5). This sequence had a 97% identity with that of *Navicula salinicola* (GenBank No. HQ396812.1). This confirmed that Navicula NBO species belonged to *Navicula salinicola*.
In order to analyze the evolutionary relationship among the Navicula NBO and other algal species, phylogenetic trees based on the 18S rDNA-V4 sequence as well as the LSU D2/D3 sequence were constructed using maximum likelihood method with 1000 bootstraps (Figure 6). Both phylogenetic trees show that the Navicula NBO was evolutionary close to *Navicula salinicola*. Thus, from these trees, we can infer that...
genetic approaches, Navicula NBO was identified as *Navicula salinicola* later on named *Navicula salinicola* NBO.

![Phylogenetic tree based on 18S rDNA-V4 (a) and LSU D2/D3 (b) sequences.](image)

*Figure 6. Phylogenetic tree based on 18S rDNA-V4 (a) and LSU D2/D3 (b) sequences.*
Navicula salinicola is classified into the genus of Navicula, the family of Naviculaceae, the order of Naviculales, class of Bacillariophyceae, phylum of Bacillariophyta, the kingdom of Chromista and the empire of Eukaryota. Three synonymous names of Navicula salinicola are Navicula incerta, Schizonema incertum, and Navicula incertata. Navicula salinicola is a ubiquitous species. Its habitat is recorded along the coast in brackish waters, in inland salt springs, in freshwaters with high electrolyte content, and in moist soils [23].

Several studies on marine Navicula salinicola have been published. One that has attracted much attention is the potential of Navicula salinicola as a source of lipids for biofuel or biodiesel production. Bogen et al. (2013) reported that among 30 microalga species from 17 genera investigated, Navicula salinicola belongs to six species that were identified with robust phototrophic growth properties, high biomass productivities equal or above 300 mg L\(^{-1}\) d\(^{-1}\) and high total lipid content equal or above 24.7% of DW. In addition, Navicula salinicola also belongs to a limited number of species that were detected to have a desirable combination of relatively high fatty acid abundances in lipids and biomass productivity [24]. Cell metabolites of marine Navicula oceanica as a source for food and medicine have also been explored such as reported by Sathasivam et al. (2019) [25]. As benthic diatoms, Navicula salinicola is widely included in studies of the marine environment, such as in experiments on the resuspension of estuarine sediments, experiments on the production of antifouling agents, and on the assessment of pollution impacts of metals and nutrients in water systems (e.g., as a biological indicator) [26]. Gao et al. (2020) have identified a novel tri-unsaturated highly branched isoprenoid (HBI) alkene from the marine diatom Navicula salinicola and determined its molecular structure. C25 highly branched isoprenoid (HBI) alkenes are common components of marine and lacustrine sediments worldwide. It could be useful as palaeoenvironmental proxies [27]. The considerable number of studies on marine Navicula salinicola related to various objectives in various fields could provide a basis for further research to study and explore the potential of Navicula salinicola NBO, a local strain from the tropical Indonesian ocean.

To analyze carbohydrate and lipid content of the Navicula salinicola NBO, total carbohydrates and lipids were isolated and characterized. Navicula salinicola NBO contained 13.13% (w/w) carbohydrates and 3.74% (w/w) lipids of dried biomass. Aslam et al. (2012) reported that marine diatoms excrete extracellular polymeric saccharides (EPS) which play an important role as cell cryoprotectants [28]. These EPS were dominant carbohydrate found in diatoms. According to Pruvost et al. (2009), not all of the total lipid fraction are equally well suited for biofuels production. Lipids containing fatty acids are preferred because they can be easily converted into biofuel by hydrotreating or into biodiesel via transesterification [24] [29]. To analyze fatty acid compositions in the total lipid of Navicula salinicola NBO, lipids were trans-esterified and analyzed by GC–FID. Navicula salinicola NBO contained SFA (saturated fatty acid), MUFA (monounsaturated fatty acid), and PUFA (polysaturated fatty acid) about 40.94, 40.00, and 19.01% (w/w) of total fatty acids, respectively (Table 1). The total lipid produced in this study was not as large as those reported in several scientific publications [24]. This might be due to different cell growth methods (e.g., medium content, growth conditions, harvesting time, etc.) as well as different lipid isolation methods. Therefore the total lipids produced are not comparable. A different case was found in the fatty acid content, wherein the total lipid of Navicula salinicola NBO had quite similar fatty acids composition as that of marine Navicula salinicola reported by Bogen et al. (2013). Lipids of both Navicula salinicola NBO and Navicula salinicola reported by Bogen et al. (2013) each contained a dominant SFA, MUFA, and PUFA of palmitic acid (C16:0), palmitoleic acid (C16:1) and eicosapentaenoic acid (C20:5) with the total concentration of 38.24, 37.23, and 12.54% (w/w of total fatty acids), respectively for the Navicula salinicola NBO (Table 1), and 27.90, 39.40, and 14.50%, respectively for the Navicula salinicola reported by Bogen et al. (2013) [24]. Stearic acid (C18:0) was not detected in Navicula salinicola NBO while only a small amount (1.90%) was found in Navicula salinicola reported by Bogen et al. (2013). SFAs with a number of C greater than 18 were not detected in both Navicula. PUFA of C18:1 was detected very small in both Navicula (Table 1) [24].
In terms of fatty acid for fuel, *Navicula salinicola* NBO produced 38.24% palmitic acid and 37.23% palmitoleic acid (Table 1). To be good biodiesel, a biofuel should have C16 or C18 fatty acids due to their similarity with aliphatic hydrocarbons in petrol [30]. According to Gopinath et al. (2010), contribution of long straight-chain fatty acids can have a significant influence on biodiesel cetane number (CN), a property that specifies the ignition quality of any fuel. A pure palmitic acid methyl ester has an average of CN of 76.6 (the second highest after stearic acid methyl ester). Thus, the higher palmitic acid content in the biodiesel, the higher CN or ignition quality of the fuel [31]. So, *Navicula salinicola* NBO fatty acids are promising sources for biodiesel.

### Table 1. Fatty acid composition (% of total fatty acids) of *Navicula salinicola* NBO

| Saturated Fatty Acids (SFA): | Number | Name                     | % (w/w) |
|------------------------------|--------|--------------------------|---------|
| C12:0                        | Lauric acid |                                | 1.86    |
| C14:0                        | Myristic acid |                             | 0.84    |
| C15:0                        | Pentadecanoic acid |                           | 38.24   |
| C16:0                        | Palmitic acid |                                 | 38.24   |
| C17:0                        | Heptadecenoic acid |                          |         |
| C18:0                        | Stearic acid |                                   |         |
| C22:0                        | Behenic acid |                                   |         |
| C24:0                        | Lignoceric acid |                                  |         |
| **Total SFA**                |         |                           | 40.94   |

| Mono unsaturated Fatty Acids (MUFA): | Number | Name                     | % (w/w) |
|--------------------------------------|--------|--------------------------|---------|
| C14:1                                | Myristoleic acid |                          | 37.23   |
| C16:1                                | Palmitoleic acid |                             | 0.79    |
| C17:1                                | Cis-10-heptadecenoic acid |                      | 1.76    |
| C18:1c                               | Oleic acid |                                    | 0.27    |
| C18:1t                               | Elaidic acid |                                     |         |
| C22:1                                | Erucic acid |                                      |         |
| C24:1                                | Nervonic acid |                                    |         |
| **Total MUFA**                       |         |                           | 40.05   |

| Poly Unsaturated Fatty Acids (PUFA): | Number | Name                                      | % (w/w) |
|-------------------------------------|--------|-------------------------------------------|---------|
| C16:3                               | Hexadecatrienoic acid |                               | 2.72    |
| C16:2                               | Hexadecadienoic acid |                                 |         |
| C18:2c                              | Linoleic acid ((9Z,12Z)-octadeca-9,12-dienoic acid) |                |         |
| C18:2n6                             | all-cis-9,12-octadecadienoic acid |                      | 0.56    |
| C18:3n6                             | Gamma-linoleic acid |                                         |         |
| C18:3                               | α-Linolenic acid |                                           | 1.03    |
| C20:3                               | Dihomo-γ-linolenic acid (Eicosatrienoic acid) |                          | 0.19    |
| C20:4                               | arachidonic acid |                                             | 0.99    |
| C20:5                               | Eicosapentaenoic acid (EPA) |                             | 12.54   |
| C22:6                               | Docosahexaenoic acid (DHA) |                                   | 0.98    |
| **Total PUFA**                      |         |                             | 19.01   |
In terms of fatty acid for nutrition, *Navicula salinicola* NBO produced 18.45% omega-3 PUFA dominated by eicosapentaenoic acid (EPA) (Table 2). As reported, marine omega-3 such as eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) are able to inhibit inflammation which links to various cardiovascular illnesses, rheumatoid arthritis, and asthma in children. Also, supplementation with omega-3 long-chain PUFA may help combat obesity, hypertension, and dyslipidemia by decreasing plasma triglycerides [32]. So, *Navicula salinicola* NBO fatty acids are also promising sources for nutritional and pharmaceutical applications.

### 4. Conclusion

NBO isolate obtained from Bokor Island, Jakarta Bay, Indonesia, showed a typical morphology and genetic property of *Navicula salinicola*. This diatom produced fatty acids that are promising candidates for both biodiesel and nutrition/pharmaceutical.

### Acknowledgments

We thank the Coordinating Ministry for Maritime and Investment Affairs of the Republic of Indonesia for financial support. Part of the work was funded by the Ministry of Research Technology and Higher Education of Republic Indonesia through the PDUPT Program in 2018.

### References

1. Tomas C R 1997 *Identifying marine phytoplankton* (San Diego: Academic Press)
2. Barsanti L and Gualtieri P 2006 *Algae: Anatomy, Biochemistry, and Biotechnology* (Florida: Taylor dan Francis Group)
3. Lavens P and Sorgeloos P 1996 *Manual on the production and use of live food for aquaculture* (Belgium: Food and Agriculture Organization of the United Nations)
4. Bhakuni D S and Rawat D S 2005 *Bioactive Marine Natural Products* (New Delhi: Anamaya Publishers)
5. Chisti Y 2008 *Trends. Biotechnol.* 26 126
6. Nurachman Z, Hartati, Anita S, Anward E E, Novirani G, Gandasasmita S, Syah Y M, Panggalbean L M G and Suantika G 2012 *Bioresour. Technol.* 108 240
7. ChunYen C, XinQing Z, HongWei Y, ShihHsin H, ChiehLun C, DuuJong L, FengWu B and JoShu C 2013 *Biochemical Engineering Journal* 78 1-10
8. De Rijk P, Van de Peer Y, Van den Broeck I and De Wachter R 1995 *J. Mol. Evol.* 41 3 366-75
9. Zimmermann J, Jahn R and Gemeinholzer B 2011 *Organic Diversity & Evolution* 11 3 173-92
10. Hamsher S E, Evans K M, Mann D G, Poulicková A and Saunders G W 2011 *Protist* 162 405
11. Andersen R A 2005 *Algal Culturing Techniques* (London: Elsevier Academic Press) p 21
12. Sambrook J, Fritsch E F and Maniatis T 1989 *Molecular Cloning: a Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press)
[6] Sanger F, Nicklen S and Coulson R 1977 Proc. Natl. Acad. 74 12 5463-67
[7] Messing J 1983 Methods in Enzymology 101 20-78
[8] DNA Sequence Assembler v4 (2013), Heracle BioSoft, www.DnaBaser.com
[9] Altschul S F, Gish W, Miller W, Myers E W and Lipman D J 1990 J. Mol. Biol. 215 3 403-10
[10] Hall T A 1999 Nucl. Acids Symp. Ser. 41 95-8
[11] Tamura K, Stecher G, Peterson D, Filipski A and Kumar K 2013 Mol. Biol. Evol. 30 12 2725-29
[12] Dubois M, Gilles K A, Hamilton J K, Rebers P A and Smith F 1956 Anal. Chem. 28 350-56
[13] Ephim E A, Sun Z F and Carrington C G 2009 Fuel 89 677-84
[14] Kusumaningtyas P, Nur baiti S, Suantika G, Amran M B and Nurachman Z 2017 Appl. Biochem. Biotechnol. 182 4 1605-18
[15] Ross R, Cox E J, Karayeva N I, Mann D G, Paddock T B B, Simonsen R and Sims P A 1979 Beihefte 64 513-33
Round F E, Crawford R M and Mann D G 1990 The Diatoms. Biology and Morphology of the Genera (Cambridge: Cambridge University Press) p 747
[16] Barber H G and Haworth E Y 1981 Guide to the Morphology of the Diatom Frustule (Freshwater Biological Association) p 112
[17] Spaulding S and Edlund M 2008 Navicula. In Diatoms of North America (Retrieved June 08, 2020, from https://diatoms.org/genera/navicula)
[18] Cox E J 2012 Journal of Phycology 48 1-31
Chen L, Weng D, Du C, Wang J and Cao S 2019 Sci. Rep. 9 7342
[19] Hale M S and Mitchell J G 2001 Aquat. Microb. Ecol. 24 287-95
[20] Juneja A, Ceballos R M and Murthy G S 2013 Energies 6 9 4607-38
[21] Nurachman Z, Hartati, Brataningtyas D S and Panggabean L M G 2012 Appl. Biochem. Biotechnol. 168 1065-75
Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M and Al Darzins 2008 The Plant Journal 54 621-39
[22] Guiry M D and Guiry G M 2020 Algaecase (Retrieved June 10, 2020, from World-wide electronic publication, National University of Ireland, Galway, http://www.algaecase.org)
[23] Bogen C, Klassen V, Wichmann J, La Russa M, Doebbe A, Grundmann M, Uronen P, Kruse O and Mussgnug J H 2013 Bioresource Technology 133 622-26
[24] Sathasivam R, Radhakrishnan R, Hashem A and Abd_Allah E F 2019 Saudi Journal of Biological Sciences 26 709-22
[25] De Jonge V N and Van den Bergh J 1987 Estuarine, Coastal and Shelf Science 24 6 725-40
[26] Targett N M, Bishop S S, McConnell O J and Yoder J A 1983 J. Chem. Ecol. 9 817-29
Belando M D, Marín A, Aboal M, García-Fernández A J and Marin-Guirao L 2017 Science of The Total Environment 574 381-89
[27] Gao S, Smik L, Kulikovskiy M, Shkurina N, Gusev E, Pedentchouk N, Mock T and Belt S T 2016 Organic Geochemistry 95 104050
[28] Aslam S N, Cresswell-Maynard T, Thomas D N and Underwood G J C 2012 Journal of Phycology 48 6 1494-509
[29] Pruvost J, Van Vooren G, Cogne G and Legrand J 2009 Bioresource Technology 100 23 5988
Aaotla H, Larmi M, Sarjoavaa T and Mikkonen S 2008 SAE Int J Engines 1 1251-62
Chisti Y 2007 Biotechnology Advances 25 3 294-306
[30] Sivakumar G, Vail D R, Xu J, Burner D M, Lay J O, Ge X and Weathers P J 2010 Engineering in Life Sciences 10 1 8-18
[31] Gopinath A, Puhan S and Nagarajan G 2010 IJEE 1 2 295-306
Knothe G, Matheaus A C and Ryan T W 2003 Fuel 82 8 971-75
Freedman B and Bagby M O 1989 Journal of American Oil Chemists’ Society 66 11 1601-05
[32] Calder P C 2012 Br. J. Clin. Pharmacol. 75 3 645-62
Lorente-Cebrián S, Costa A G, Navas-Carretero S, Zabala M, Martinez J A and Moreno-Aliaga M J 2013 J. Physiol. Biochem. 69 3 633-51