Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2016, 8(12):110-117
(http://derpharmachemica.com/archive.html)

Identification of Blue-Green Algae *Uncultured Oscillatoria sp IPOME-4* Isolated from Local Industry Effluent with The Potential as β-Carotene Feedstock

Widiyanti Sekatresna, Abdi Dharma, Rahmiana Zein and Zulkarnain Chaidir

Department of Chemistry, Faculty of Mathematics and Natural Sciences, Andalas University, Padang, 25163, Indonesia

---

ABSTRACT

Cyanobacteria which is also known as blue-green algae have gained considerable attention as they offer high value bioactive compounds with feasible application for pharmaceutical and nutraceutical products. In this study, a filamentous cyanobacteria has been isolated from Palm Oil Mill Effluent (POME) of local industry. Morphological observation and biomolecular analysis were performed to identify the isolated species. The molecular phylogenetic analysis was based on the sequences of 16S rRNA gene. β-carotene content and dry biomass main components (carbohydrate, protein and lipid) were also investigated. The result of biomolecular analysis, supported by morphological identification confirmed that the strain was Uncultured oscillatoria sp. Carbohydrate, protein and lipid contents of the strain were 15.301%, 54.384% and 11.75% respectively. Under two different culture conditions (culture incubated in normal BBM medium and in nitrogen depletion medium), β-carotene content for both cultures were 0.697 % and 0.842 % respectively. The increase of β-carotene content in nitrogen depletion medium indicated that the stress applied is effective enough to enhance β-carotene production.

Key words: cyanobacteria, 16S rRNA, phylogenetic analysis, HPLC analysis, β-carotren

---

INTRODUCTION

An increasing need for high value compounds with the guarantee for safer, environmentally friendly and other positive issues including incompatible with food supply has placed microalgae as the most interesting feedstocks for different kinds of bioproducts. Microalgae are eukaryotic or prokaryotic photosynthethic microorganisms that use solar energy, nutrients and CO_2 to produce a wide range of important biochemicals. These microorganism have several applications such as in food, pharmaceutical, cosmetic and biofuel products. Cyanobacteria are of prokaryotic one and also known as blue-green algae. They are found worldwide in diverse habitats either freshwater, marine, terrestrial or even extreme environments [1]. Cyanobacteria exist in different kinds of features including unicellular, filamentous, planktonic, benthoic and colonial ones [2]. The term blue-green algae as other name for cyanobacteria is caused by the presence of phycocyanin and phycoerythrin which belong to phycobiliprotein (water-soluble chromoproteins) and have the association with photosynthetic apparatus[3]. Cyanobacteria have the ability to fix atmospheric nitrogen (N_2). Using nitrogenase enzyme, molecular nitrogen is reduced to ammonia in the presence of hydrogen. This characteristic is unique for cyanobacteria, making this microalgae are not limited by nitrogen [4]. Several cyanobacterial strains have been explored for their valuable products. One of the most well

known edible blue-green algae with protein-dence food is *Spirulina (Arthrospira)*. It contains amino acid profile which is considered as high biologic-value protein. It is also rich in vitamin B12, beta-carotene, iron, calcium and phosphorous [5].

In recent years there have been considerable interest in the potential of cyanobacteria as sources of various bioactive compounds since these compounds are potentially applied in a commercial scale such as in agriculture, nutraceutical, effluent treatment as well as in biofuel production [2]. These compounds have been proved to be active towards bacteria, virus and other phatogenic microorganisms due to pharmaceutical and nutraceutical significances they offer, thus reduce the risk of dangerous diseases. Some of the most interesting chemicals produced by several strains of cyanobacteria are pigments, vitamins and enzymes [2]. Cyanobacterial pigments are widely used as nutritional ingredients and natural dyes for foods and cosmetics [6]. Carotenoids, a group of natural pigments which exist in phytoplankton, marine algae, plants and some species of bacteria have the ability to yield a variety of colours ranging from yellow to reddish brown [7]. Carotenoids which contain high reactive conjugated double bonds have been kown to be effective in trapping free radicals and at the same time function as antioxidants in quenching toxic radicals [8] Carotenoids in cyanobacteria play important role in light harvesting process, photoprotection and structure organization [9]. The mechanisms performed by carotenoids are beneficial to protect human against infections and degenerative diseases such as atherosclerosis, cardiovascular disease (CVD), cancer, inflammatory, neurological diseases and diabetes [10]. Some studies have successfully characterized and extracted carotenoids contained in microalgae [11]-[13]. The carotenoid most commonly used in industry is β-carotene[14]. The application of β-carotene among others are in food, cosmetic and pharmaceutical industries, and especially used as colorants, antioxidants and anti cancer agents. It also provides dietary source of vitamin A [15].

This study was intended to identify a blue-green algae species isolated from Palm Oil Mill Effluent (POME) along with exploring its potential for β-carotene production. As growth environment naturally influence the characteristics of species, it is expected the isolated species would display different characteristic from already known species with promising β-carotene content . Identifications include morphological and biomolecular analysis accompanied by determination of β-carotene content and biomass main components (carbohydrate, protein and lipid).

MATERIALS AND METHODS

Isolation

Isolation was carried out along with three other eukaryotic isolates from the same Palm Oil Mill Effluent (POME) sample provided by Mutiara Agam Company, West Sumatera, Indonesia as reported by Abdi Dharma et al [16]. Method of isolation was serial dilution in which 1 mL sample was diluted in 9 mL BBM medium with dilution of 10^{-1} to 10^{-10}. The culture was incubated at room temperature with the illumination of sunlight captured inside the laboratorium and equipped with bubbling air from an aerator. Regular subculturing was carried out until pure culture was obtained.

Morphology Analysis

Morphology analysis was performed to identify the feature and structure of the strain. Identification consisted of optical microscopy observation, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis. The feature of the strain was observed under Binocular Olympus Microscope. Further identifications were performed by SEM and TEM instruments to analyze more absolute structure. For SEM analysis, briefly biomass of the isolated strain was spread on an specimen holder which was coated by double sticky tape and cleaned with hand blower. Sample was than placed in the specimen chamber and observed by SEM instrument (SEM Hitachi S-3400). While for TEM analysis in a brief, dry biomass of the species was dispersed in a special solvent, after a series of sample preparation, sample was observed under TEM Instrument (TEM-JEM-1400).

Biomolecular Analysis

Genomic DNA was extracted from the strain IPOME-4 using DNeasy Plant Mini Kit (Qiagen). After electrophoresis process in BIO-RAD Electrophoresis Chamber and Power Pac apparatus, amplification of ribosomal DNA was performed by PCR 16S-rRNA Cyanobacteria using primers of CYA106F (5'-CGGACGGGTGAGTAACGCGTGA-3') and GYA781R (5'-GACTACWGGGGTATCTAATCCCWTT-3') [17]. PCR amplification was conducted on BIO-RAD C1000 Thermal Cycler apparatus with an initial denaturation of DNA at 94^{o}C for 5 min followed by 35 cycles of denaturation at 94^{o}C for 1 min, annealing at 62^{o}C for 30 s and extension at 72^{o}C for 1 min, then final extension at 72^{o}C for 5 min, and 4^{o}C hold. PCR products were resolved on

1,5 % agarose gel, stained with Gel-Red and visualized in GelDoc BioRad. After purification with Na Acetic methods, PCR products were sent to Macrogen, South Korea for sequencing. Sequencing datas were edited using Geneious 7.0.6 software. Then BLAST (Basic Local Alignment Search Tool) was used to compare the 16S-rRNA sequences of the strain with sequences in NCBI GenBank database. Based on the Neighbor-Joining (NJ) method, phylogenetic tree was constructed using Geneious Tree Builder.

Growth Rate and Biochemical Analysis

Growth Rate analysis : Prior to biochemical analysis, cell growth was determined by daily measuring the optical density (OD) at 680 nm with UV-VIS Spectrophotometer (Genecys 20). The datas were subsequently converted to dry cell weight (dcw) using calibration curve prepared in advance.

Carbohydrate Content : Carbohydrate content was analyzed based on the Phenol-sulfuric acid method [18] with little modification, in which 1 mL of microalgae culture was mixed with 1 mL of 5 % phenol solution in a test tube followed by addition of 5 mL concentrated sulfuric acid. After 10 min of incubation the mixture was vortexed for 30 s, then placed in a water bath at temperature of 30^{o}C for 20 min. The absorbances were measured at 490 nm. Along with sample, a series of glucose standard solution were prepared with similar treatment as sample.

Protein Content : Protein content of the isolate was determined based on Lowry method by preparing sample solution and BSA standard solution following the instruction of the procedure [19]. The absobances were measured at 750 nm.

Lipid Content : Determination of lipid content was performed according to Bligh and Dyer method [20] with some modification. Briefly, the dry biomass of strain was blended with 4 mL chloroform and 2 mL methanol. After incubating for 12 h while shaking at 200 rpm/m, supernatant was separated from biomass by centrifugation. The chloroform layer was recovered and dried in an oven at 50^{o}C. Lipid content was calculated gravimetrically.

HPLC analysis of β-Carotene Content

Prior to analysis, two cultures with different condition were prepared, one culture contained strain with normal BBM medium while the other one is strain cultured in nitrogen depletion medium. For the second one, preparation was performed by transferring the culture at the end of exponential phase from normal nutrition medium into nitrogen depletion medium. Cultures were harvested at the stationary phase. Biomass of the cultures were dried up, then 100 mg of dried biomass was extracted with acetone. To separate the cell debri the solution was centrifuged for 3 m at 4000 rpm. The solvent was dried up and the remaining dried carotenoid sample was dissolved in 100 µL mobile phase which consisted of dichloromethane, acetonitrile and methanol with the ratio of 20:70:10 (v/v/v/) [21]. A series of β-carotene standard solution was prepared and treated with the same procedure as sample solution. Both β-carotene standard and sample solution were injected into HPLC system (Shimadzu SPD-20A/SPD-20AV equipped with Shim-Pack VP-ODS, LUNA C18 or equivalent ODS column (150 x 4.6 mm i.d., 5µm) and measured at 450 nm.

RESULTS AND DISCUSSION

Morphological Characterization

Morphological feature of the strain IPOME-4 observed under optical microscope demonstrate that the cells are homogenous in shape with similar sizes (fig. 1a). Based on the characteristic of the cells in which they exist in unbranched filamentous cells, it can be inferred that the strain belongs to genus *Oscillatoria.* Unlike common *Oscillatoria* which mostly posses long filaments, this one has shorter filaments. Under normal BBM condition the microalgae appeared as bright bluish green cells with apical cells were observed at the end of filaments (Fig. 1b). When grown under nitrogen depletion condition the cells turn yellow and the cell size appeared to be a little bigger (Fig. 1c).

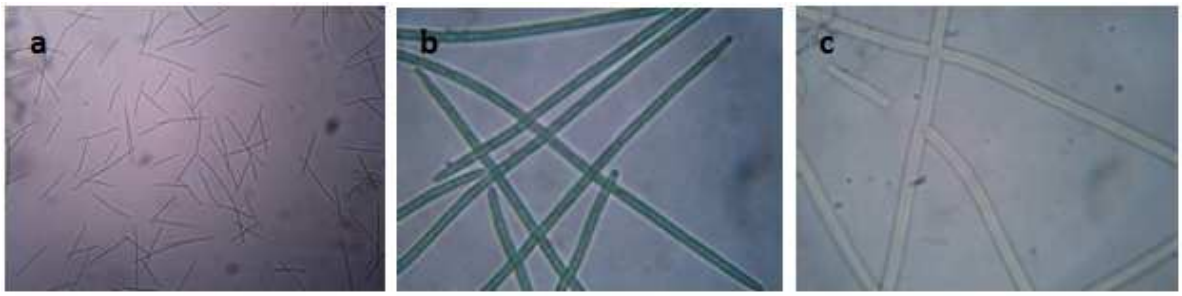


Figure 1 : Features of strain IPOME-4 under optical microscope observation (a). with magnification 100x, (b). cultured in normal BBM medium with magnification 1000x, (c). cultured in nitrogen depletion medium with magnification 1000x
SEM analysis of strain IPOME-4 is displayed in figure 2

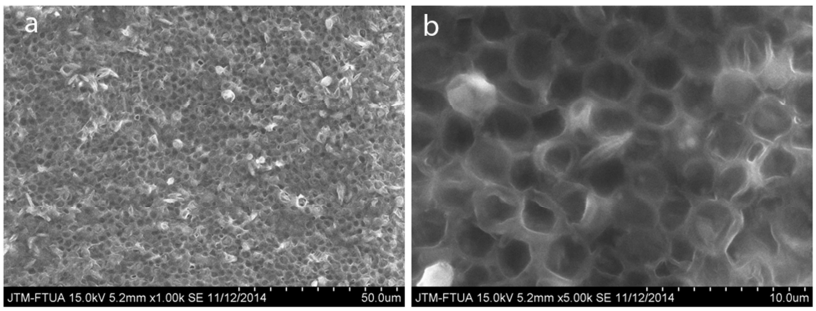


Figure 2 : Strain IPOME-4 feature under SEM observation, (a) at magnification 1000x and (b) at magnification 5000x

SEM photograph gives specific feature of the cell wall surface. It is usually unique for individual species and there is similarity for some species within the same genus as reported by Abdi Dharma et al [16]. The image of SEM identification displayed specific pattern of strain IPOME-4 cell wall. Pores like structure can be observed dominantly occupying the whole surface.

TEM analysis of strain IPOME-4 is presented in figure 3

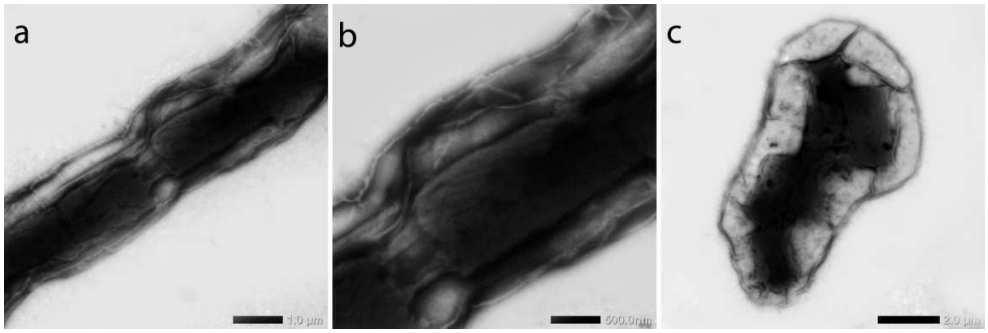


Figure 3 : Morphology of strain IPOME-4 under TEM observation

Parallel images of the strain were shown in fig. 3a and 3b, while cross-sections image was displayed in fig. 3c. Filament sheath, the outer layer of the cell can be observed especially in the cross-sections image covering the dark compact of cytoplasm (fig. 3c). Cell wall and plasma membrane are also observed, while other parts of the cell can not be seen clearly.

Biomolecular analysis

The strain IPOME-4 was characterized using 16S rRNA gene analysis and its phylogenetic relationship with 16 others of cyanobacteria species is available in database. Phylogenetic tree was constructed based on the datas (fig. 4). BLAST analysis and phylogenetic tree that produced using Geneious Tree Builder and based on Neighbor-Joining

(NJ) method gave the result that strain IPOME-4 has highest similarity with four other species namely *Oscillatoria acuminate NTDM04, Nostoc sp. TPCR9, Uncultured Cyanobacterium clone D57C23* and *Uncultured Oscillatoria sp* with sequence identity of 98%, 97%, 94% and 98 % respectively. The matrix and phylogenetic tree resulted from Neighbor-Joining (NJ) method showed that the closest distance was with *Uncultured Oscillatoria sp*. It is also supported by morphological analysis which demonstrates that the structure of strain IPOME-4 is markedly different from *Oscillatoria acuminate NTDM04, Nostoc sp. TPCR9* and *Uncultured Cyanobacterium clone D57C23*. In case of strain *Oscillatoria acuminate* NTDM04, although the strain IPOME-4 posseses a high (98%) sequence identity with *Oscillatoria acuminate* NTDM04, both species have different morphological features. *Oscillatoria acuminate NTDM04* has long and interwoven filaments, while strain IPOME-4 is characterized with short and rather straight filaments. Thus it can be inferred that the closest similarity of strain IPOME-4 is with *Uncultured oscillatoria sp.* The strain has been deposited into The National Center for Biotechnology Information (NCBI) under accession number of **KX033860**.

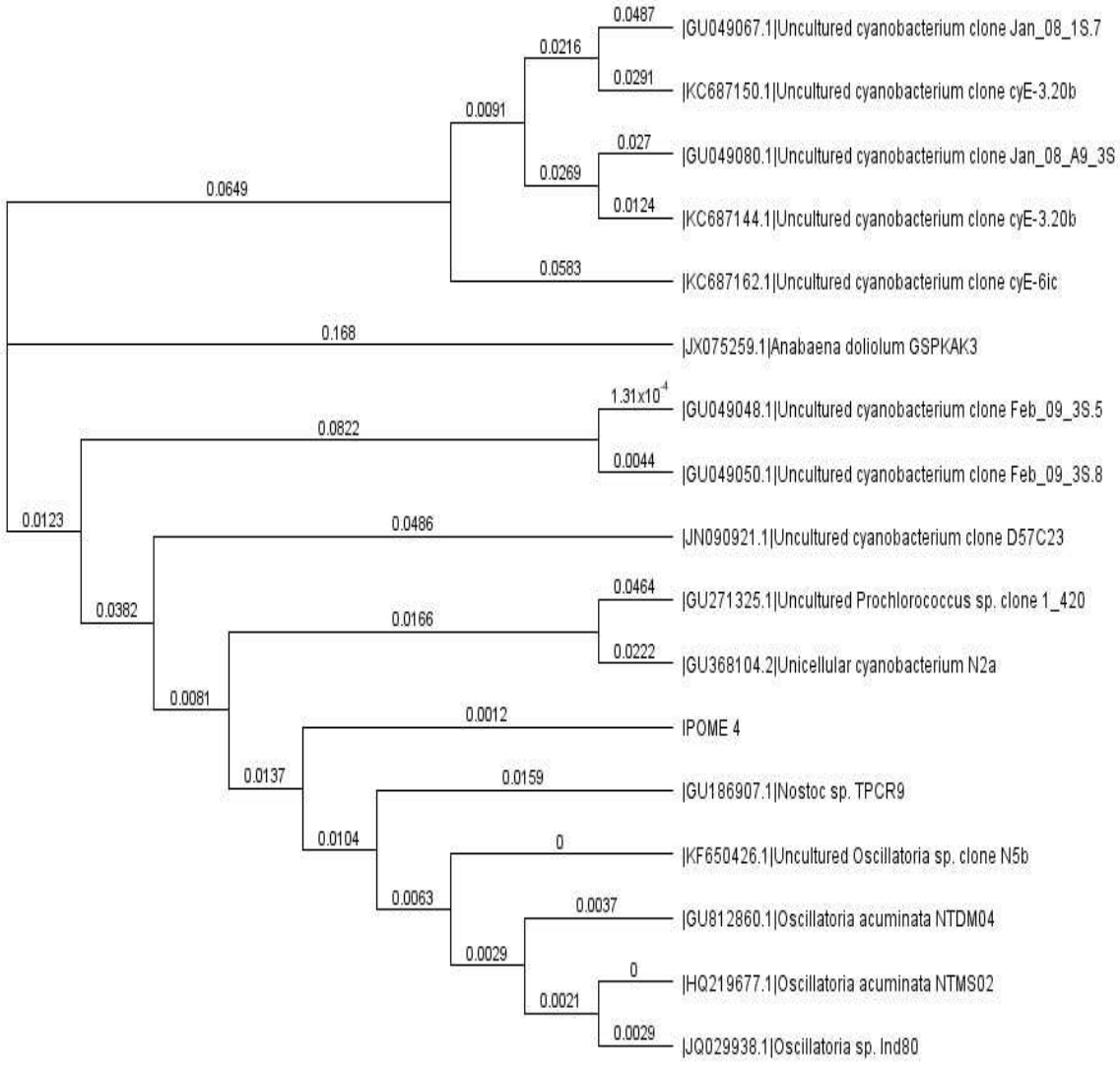


Figure 4. Phylogenetic tree of strain IPOME-4 based on 16S rRNA gene sequences

Biochemical Composition

The biomass main components of strain *Uncultured oscillatoria sp IPOME-4* which consist of carbohydrate, protein and lipid is shown in table 1.

Table 1 : Biomass Composition of strain *Uncultured oscillatoria sp IPOME-4*

Main Components of Biomass (%)		
Carbohydrate	**Protein**	**Lipid**
15.301 ± 1.058	54.384 ± 1.501	11.750 ± 0.866

The datas obtained from determination of main composition of the strain indicated that the highest content is protein, which accounts for 54.38 %. The figure is quite significant and could be considered to be relatively high as compared to protein content of well known *Spirulina sp* which contains 60-70% of its dry weight [22]. Microalgae with significant protein content have the potential to be explored as the source of nutritious products. Thus it is suggested, further study should be carried out for this possibility. The second major component of strain *Uncultured oscillatoria sp IPOME-4* is carbohydrate with percentage of 15.30 %. While lipid content of the strain is relatively low (11.75% of dry cell weight).

HPLC Analysis for β-Carotene

Determination of β-carotene content was intended to investigate the potential of strain *Uncultured oscillatoria sp IPOME-4* as source of β-carotene under two different culture conditions. One culture was incubated under normal nutrition medium, and the other one was the culture with nitrogen depletion medium. Figure 5 displays the two cultures. Under normal BBM medium, the culture has bright green colour, while the colour of the culture with nitrogen depletion medium after 7 days of incubation gradually turned into yellow.

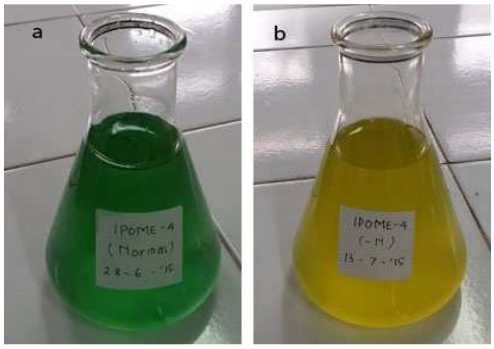
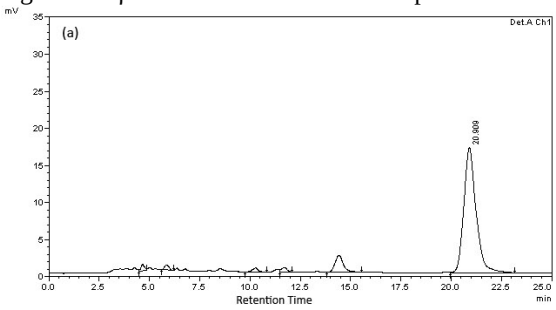


Figure. 5. Cultures of strain *Uncultured oscillatoria sp IPOME-4* (a) under normal nutrition medium, and (b) under nitrogen depletion medium

Fig. 6(a, b and c) show chromatograms of β-carotene standard and sample solutions.



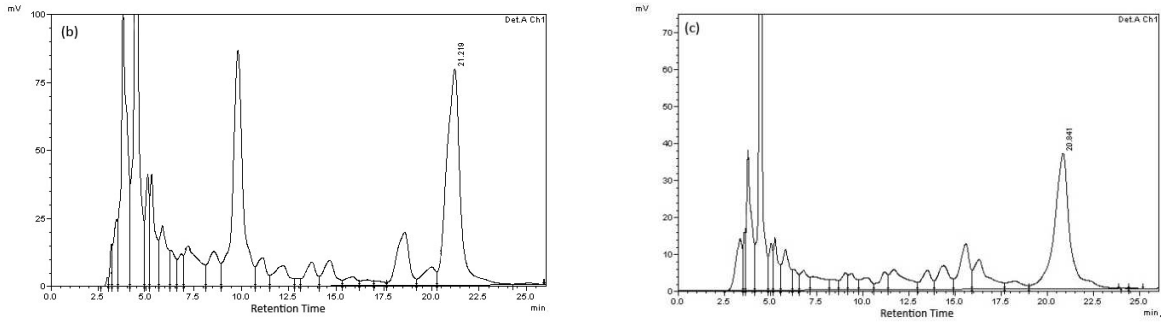


Figure 6. HPLC chromatograms of β-caroten for (a) standard solution, (b) sample with normal nutrition medium (after 10x dilution), and (c) sample with nitrogen depletion medium (after 20x dilution)

HPLC analysis above demonstrate that strain *Uncultured oscillatoria sp IPOME-4* either under normal nutrition medium or with nitrogen depletion medium had peaks at almost the same retention time as peak of β-carotene standard which indicated both samples contain β-Carotene. Based on HPLC peak area and the standard curve constructed from measurement of a series of β-carotene standard solutions, β-carotene in sample solutions can be calculated. β-carotene contents for both samples are 0.697 % and 0.842 % % (dry cell weight) respectively. The results are in the range of average content of carotenoids in most algae (0.1-2% of dry cell weight) [23]. Comparing the two results, there is an increase of β-Carotene content after the culture was introduced to nitrogen depletion medium. It was also confirmed by yellow colour of the culture. The change in colour may be due to the increase of β-carotene under stress condition in accordance with the function of β-carotene as the protective accessories for the cell in order to maintain the continuation of cell growth under undesirable condition [24]. Although the content is moderately significant, however the strain *Uncultured oscillatoria sp IPOME-4* is quite potential to be applied in a large scale. For this purpose, further investigation should be carried out on determination of the optimum nitrogen concentration in nitrogen depletion medium. Moreover, it has to be noticed that there are still any other approaches can be applied to improve β-caroten content of the strain, among others are light irradiation, salinity and other nutrient limitations. These treatments would lead towards finding the most suitable approach for optimal β-carotene production

CONCLUSSION

Morphologycal and biomolecular identifications of the isolated species confirmed that the blue-green algae species isolated from Palm Oil Mill Effluent of local industry was strain *Uncultured Oscillatoria IPOME-4.* The composition of carbohydrate, protein and lipid are 15.30%, 54.38% and 11.75% respectively. With high protein content, there is possibility for the strain to be further explored as source of nutritious products. The HPLC analysis of β-carotene revealed that the strain also contain 0.697 % and 0.842 % % of dry cell weight for respective culture condition tested. The result was quite significant in correlation with the average content of β-carotene in most microalgae. It is suggested to apply any other approaches to find the best treatment for highest β-carotene content.

REFERENCES

[1] A. Ramsy, Q.Antonio., *Toxins*, **2014**, *6*, 1929-1950
[2] L. Nyok-Sean, M. Minami, A. Amirul Al-Ashraf., *BioMed Research International*, **2015**, 1-9
[3]C. Lon, N. Tandeau Marsact, B. G. cohen., *Proc. Nat. Acad. Sci. USA,* **1973**, 11, 3130-3133
[4]O. Nweze N., *Plant Product Research Journal***, 2009**, 13, 8 – 14
[5]S. Gabriela Gutiérrez, C. Luis Fabila, C. Germán Chamorro., *Nutr Hosp*, **2015**, 32, 34-40
[6] A.E. Azza M, M. Amal A, S. Farag A., *Journal of Applied Pharmaceutical Science*, **2014,** 4 (07), 069-075
[7] V. Joa˜o C, P. Hugo, V. Marta, L. Rosa., *Photosynth Res*, **2015**, 1-14
[8]A. M. Nauman, M. Saleemullah, S. Hamid Ullah, K. Iqtidar A., A.U.R. Saljoqi., *Sarhad J. Agri*, **2007**. 23(3), 767-770
[9]B. Rudi, S.Ivo H.M., K. John T.M., G. Rienk., D.Jan P., *Chemical Physic*, **2010,** 1-6
[10]J.R. Maria Filomena, M. Alcina Maria M.B, M. Rui Manuel S.C., *Mar. Drugs* **2015**, *13*, 5128-5155
[11]G. Borhane Samir, C. Samira, K. Douadi, S. Ben, J. Clayton, A. Spiros N., *Biomass and Bioenergy*, **2014**, 69, 265-275

[12]S**.** Chitta Ranjan, D. Lima, B. Bagmita, G. Bhabesh Ch., *Asian Journal of Plant Science and Research*, **2012**, 2(4), 546-549
[13]C.Lourdes Casas, S. Casimiro Mantell, R. Miguel Rodríguez, D.O. Enrique J. Martínez., *American Journal of Analytical Chemistry*, **2012**, 3, 877-883
[14]M. Kamla, T. Jayanti, G. Sneh., *International Journal of Microbial Resource Technology*, **2012**, 1(4), 361-365
[15]D. D. Selvi, K. Dhandayuthapani., *Int.J.Curr.Microbiol.App.Sci*, **2013**, 2(3), 37-43
[16]S**.** Widiyanti, D. Abdi, Z. Rahmiana, C. Zulkarnain., *Journal of Chemical and Pharmaceutical Research*, **2015**, 7(9), 222-231
[17]B. Ulrichnu, G. P. Ferran, M. Gerard., *Applied And Environmental Microbiology*, **1997**, 63(8), 3327–3332
[18] A. Ammar A., B. Asmeret Asefaw, G. Teamrat A., *Carbohydrate Polymer*, **2013**, 97, 253-261
[19] L. Cynthia Victoria González, G. María del Carmen Cerón, F. Francisco Gabriel Acién, B. Cristina Segovia,C. Yusuf, S. José María Fernández., *Bioresource Technology*, 2010. 101, 7587-7591
[20]Y. Cheng, L. Junhan, F. Yong, R. Xiaohui, H. Guangrong, L. Fuli., *Biotechnology for Biofuels*, **2011**, 4(47), 1-8
[21]S. Bhagavathy, P. Sumathi., *Biomedicine & Preventive Nutrition*, **2012**, 2, 276-282
[22]T. Guangwen, S. Paolo M., *Journal of Pharmacy and Nutrition Sciences*, **2011**, 1, 111-118
[23]M. John J., *Rev Environ Sci Biotechnol*, **2011**, 10, 31-41
[24]P. Dipak S, S.S. Lele., *Indian Journal of Biotechnology*, **2005**, 4, 476-483