Characterization of a Korean Domestic Cyanobacterium *Limnothrix* sp. KNUA012 for Biofuel Feedstock

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A filamentous cyanobacterium, *Limnothrix* sp. KNUA012, was axenically isolated from a freshwater bloom sample in Lake Hapcheon, Hapcheon-gun, Gyeongsangnam-do, Korea. Its morphological and molecular characteristics led to identification of the isolate as a member of the genus *Limnothrix*. Maximal growth was attained when the culture was incubated at 25°C. Analysis of its lipid composition revealed that strain KNUA012 could autotrophically synthesize alkanes, such as pentadecane ($C_{15}H_{32}$) and heptadecane ($C_{17}H_{36}$), which can be directly used as fuel without requiring a transesterification step. Two genes involved in alkane biosynthesis−an acyl-acyl carrier protein reductase and an aldehyde decarbonylase−were present in this cyanobacterium. Some common algal biodiesel constituents−myristoleic acid ($C_{14:1}$), palmitic acid ($C_{16:0}$), and palmitoleic acid ($C_{16:1}$)−were produced by strain KNUA012 as its major fatty acids. A proximate analysis showed that the volatile matter content was 86.0% and an ultimate analysis indicated that the higher heating value was 19.8 MJ kg^{-1}. The isolate also autotrophically produced 21.4 mg g^{-1} phycocyanin−a high-value antioxidant compound. Therefore, *Limnothrix* sp. KNUA012 appears to show promise for application in cost-effective production of microalga-based biofuels and biomass feedstock over crop plants.

Key words : Alkane-producing genes, biofuel, cyanobacteria, phycocyanin, potential feedstock

Introduction

Renewable and sustainable energy resources have received much renewed interest as a solution to the heavy reliance on fossil fuels since global petroleum supplies have diminished and serious environmental problems have arisen from greenhouse gas emissions. Recently, photosynthetic microorganisms have gained particular interest as a new source for biofuel feedstock because they are able to convert carbon dioxide (CO_2) to a variety of potent biofuels [3, 7, 11]. In particular, microalgae have become an attractive candidate for liquid transport fuel production due to their higher photosynthetic efficiency and oil yield compared to terrestrial energy crops [12, 16].

However, there are still a number of technical barriers that must be overcome and one of the major challenges in algae-based biodiesel production is to reduce the cost of lipid extraction that accounts for up to 90% of the total energy consumption [15]. Algal biodiesel is mainly obtained by transesterifying algal oil triglycerides with an alcohol in the presence of catalysts [13, 17]. Methanol is the most commonly used alcohol in this process due to its low cost [6], yet it is also responsible for 26% of the total energy consumption in biodiesel production [5]. It has been reported that some freshwater and marine cyanobacteria could naturally produce alkanes such as pentadecane ($C_{15}H_{32}$) and heptadecane ($C_{17}H_{36}$) [25, 26]. The biological function of alkane in cyanobacteria is still unclear, but it was hypothesized that the hydrocarbons blended into the lipid bilayers may enhance permeability, flexibility, and fluidity against curvature and oxidative stresses [32]. As petroleum-based diesel is made up of a mix of alkanes with 8-20 carbon atoms, these cyanobacteria-derived alkanes can be directly used as biodiesel without converting triglycerides to liquid hydrocarbons and thus may serve as a possible

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candidate to replace fossil fuels.

In this study, a filamentous cyanobacterium, *Limnothrix* sp. KNUA012 was axenically isolated from a summer bloom sample in Lake Hapcheon, Korea and its potential as biofuel feedstock was investigated.

Materials and Methods

Sample collection and isolation

Bloom samples were collected in September 2010 from Lake Hapcheon, Bongsan-ri, Bongsan-myeon, Hapcheon-gun, Gyeongsangnam-do (35° 37′N, 128° 02′E). Samples were then taken to the laboratory and 1 ml aliquots of these samples were inoculated into 100 ml BG-11 medium [21] with cycloheximide (Sigma, St. Louis, MO, USA) at a concentration of 250 μg ml^{-1}. The flasks were incubated at 25°C with shaking at 160 rpm on an orbital shaker (VS-202D, Vision Scientific, Bucheon, Korea) until cyanobacterial growth was apparent. Well-grown cyanobacterial cultures (1.5 ml) were centrifuged at 3,000 g for 15 min (Centrifuge 5424, Eppendorf, Hamburg, Germany). Resulting pellets were streaked onto BG-11 agar and filamentous growth was monitored daily. When emerging cyanobacterial filaments were macroscopically visible, they were aseptically transferred to fresh BG-11 plates to separate cyanobacteria from contaminating bacteria. Cyanobacterial filaments were then streaked onto R2A and LB agar plates (Becton, Dickinson and Company, Sparks, MD, USA) and incubated in the dark to check the axenic status of the culture for 14 days. The stain obtained in this study was deposited in the Korean Collection for Type Cultures (KCTC) under the accession number KCTC 12064BP.

Morphological and molecular identification

The isolate was grown in BG-11 medium for 20 days. Live cells were harvested by centrifugation at 3,000× g for 5 min, washed twice with sterile distilled water, and examined at 1,000× magnification under a Nikon Eclipse E100 Biological Microscope (Tokyo, Japan). For molecular analysis, genomic DNA was extracted using a DNeasy Plant Mini kit (Qiagen, Hilden, Germany). For the amplification of 16S rRNA gene fragments, the primer set, CYA106F and CYA781R(a) and CYA781R(b) described by Nübel *et al*. [20] was used. Due to the highly conserved nature of the 16S rRNA gene, three other genetic markers, the phycocyanin encoding operon intergenic spacer (PC-IGS), ribosomal 16S-23S intergenic spacer (ITS), and RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) *rbc*LX, were employed. The PC-IGS was amplified using the primer pair, PCβF and PCαR specific for cyanobacteria [19]. The ribosomal 16S-23S ITS region was evaluated using primers, 16S1407F and 23S30R [30] and region RuBisCO *rbc*LX was also amplified with primers, CW and CX, described by Rudi *et al*. [23], respectively. DNA sequences obtained in this study were deposited in the database of the National Center for Biotechnology Information (NCBI) under accession numbers JQ653272, KU297785, KU662322, and KU662323 (Table 1).

Phylogenetic analysis using 16S rRNA gene

The sequence obtained from this study was compared to 16S rRNA gene sequences in the NCBI GenBank database using the BLASTN algorithm [1]. Its closely related *Limnothrix* sequences were downloaded and aligned in the Molecular Evolutionary Genetics Analysis (MEGA) software, ver. 6.0 [29], with the ClustalW tool. The best-fit nucleotide substitution model (K2) was selected by means of MEGA ver. 6.0 on the basis of the Bayesian information criterion. This model was used to build a maximum likelihood (ML) phylogenetic tree with 1,000 bootstrap replicates [8]. *Synechococcus elongatus* PCC 6301 (NR_074309) was used as an outgroup.

Temperature testing

Late-exponential phase cultures of *Limnothrix* sp. KNUA

Table 1. Results from BLAST searches using the sequences of the 16S rRNA, PC-IGS, 16S-23S ITS, and *rbc*LX genes of strain KNUA012

Marker gene	Accession No.	Length (bp)	Closest match (GenBank accession No.)	Query cover (%)	Sequence similarity (%)
16S rRNA	JQ653272	662	*Limnothrix* sp. B15 (GQ848190)	99	100
PC-IGS	KU297785	680	Uncultured cyanobacterium clone DC07PC-38 (HM020449)[a]	94	100
16S-23S ITS	KU662322	632	*Geitlerinema unigranulatum* BCCUSP 94 (KJ735459)	82	96
*rbc*LX	KU662323	1,082	*Limnothrix planktonica* KLL-C001 clone (KP698043)	23	94

[a]Closest cultured match: *Geitlerinema unigranulatum* BCCUSP94 (FJ545642)

012 (1 ml each) were inoculated into BG-11 medium in triplicate and incubated for 20 days. Survival and growth of KNUA012 cells maintained at temperatures ranging from 5°C to 25°C (at intervals of 5°C) were examined to determine the optimum culture temperature. The cell biomass was separated from the bottom of the flask and thoroughly mixed by pipetting and samples taken from each time point were homogenized by sonication for 15 sec on an ultrasonic cell disruptor (Model 550, Fisher Scientific, Pittsburgh, PA, USA). Cyanobacterial cell density was determined by measuring the optical density (OD) of the cultures at 750 nm with an Optimizer 2120UV spectrophotometer (Mecasys, Daejeon, Korea).

Gas chromatography/mass spectrometry (GC/MS) analysis

The isolate was autotrophically grown in BG-11 medium for 20 days under its optimal conditions and cells were harvested for lipid analysis. The samples were freeze-dried to enhance the extraction efficiency. Then, 100 mg of each dried sample was blended with a mixture of chloroform:50% methanol (1:1) and extracted for 16 hr at 25°C. After extraction, the chloroform extract was isolated and dried in a rotary evaporator. The dried extract was treated with a pre-made solution of methanol and potassium hydroxide to facilitate transesterification. Next, 2 ml of hexane was added to the mixture to isolate the fatty acid methyl esters (FAMEs). The whole mixture was heated to 30°C and stirred for 10 hrs. The mixture was then cooled and the methanol and hexane layers were separated. The yellow hexane layer was isolated for further analysis. GLC-90 (Supelco, Bellefonte, PA, USA) was used as external standard to calculate the FAME concentrations. The FAME composition was analyzed using a 6890N gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with a 5973N mass selective detector and a HP-5MS capillary column (30 m ×0.25 mm ID ×0.25 μm film thickness). The initial oven temperature of the gas chromatograph was 120°C. It was maintained for 2 min, increased to 300°C at a rate of 5°C min^{-1}, and held for 22 min. The injection volume was 1 μl with a split ratio of 20:1. Helium was used as carrier gas at a constant flow rate of 1 ml min^{-1}. The mass spectrometer parameters were as follows: injector and source temperatures were 250°C and 230°C, respectively, and the electron impact mode at an acceleration voltage of 70 eV was used for sample ionization, with an acquisition range

from 50-550 m z^{-1}. Wiley/NBS libraries were used as reference databases.

Biomass characterization

The remaining freeze-dried biomass samples were pulverized with a mortar and pestle and sieved through ASTM No. 230 mesh (opening = 63 μm). Ultimate analysis was conducted in order to determine the carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) contents using a Flash 2000 elemental analyzer (Thermo Fisher Scientific, Milan, Italy). Higher heating value (HHV) was estimated by the following equation developed by Friedl et al. [9]: [HHV = $3.55C^2$ − 232C - 2,230H + 51.2C × H + 131N + 20,600 (MJ kg^{-1})].

Proximate analysis was carried out on a DTG-60A thermal analyzer (Shimadzu, Kyoto, Japan). Platinum pans were used to contain 30 mg of α-alumina (α-Al$_2$O$_3$) powder (Shimadzu, Kyoto, Japan) as a reference material and approximately 10 mg of each sample, respectively. Nitrogen (>99.999%, N$_2$) was supplied as the carrier gas at a rate of 25 ml min^{-1} to protect the microalgae powder from oxidation. Samples were heated from 50°C to 900°C at a rate of 10°C min^{-1}. Thermogravimetric analysis (TGA) data were analyzed by ta60 Ver. 2.21 software (Shimadzu, Kyoto, Japan).

Phycocyanin (PC) concentration in the cyanobacterium was quantified by a slightly modified method of Silveira et al. [28]. Briefly, 0.16 g of dried biomass in 2 ml of distilled water (at a biomass-solvent ratio of 0.08 g ml^{-1}) was mixed with shaking at 100 rpm on an orbital shaker at 25°C for 4 hr. Then, the tubes were centrifuged at 10,000 g for 15 min and the OD of the supernatant was measured at 615 and 652 nm. The PC concentration was calculated by the following equations: [PC (mg ml^{-1}) = (OD$_{615}$−0.474 × (OD$_{652}$)) / 5.34]. The extraction yield was estimated by the following formula: [Yield (mg g^{-1}) = PCC × V / DB], where V is the volume of solvent (ml) and DB is dried biomass (g).

Detection and identification of genes involved in alkane biosynthesis

Two primer sets were designed according to the fully sequenced Nostoc sp. PCC 7120 genome to amplify an acyl-acyl carrier protein (ACP) reductase and an aldehyde decarbonylase in Limnothrix sp. KNUA012. Partial fragments of both genes in Limnothrix sp. KNUA012 were successfully amplified by the primer sets, alr5284F and Lim_red_4R, and

ADDP1F and ADDP6R, respectively. The downstream and upstream regions of the known sequences were further obtained using genome walking-PCR based on the manufacturer's protocol (Clontech, Mountain View, CA, USA). Finally, the full length acyl-ACP reductase and aldehyde decarbonylase including open reading frames (ORFs) in *Limnothrix* sp. KNUA012 were amplified by the primer sets, KNUA012_RED_F and KNUA012_RED_R, and KNUA012_aldehyde_decarbonylase_F and KNUA012_aldehyde_decarbonylase_R, respectively. All the primers used in this study were designed by using Primer3Plus software [31] and listed in Table 2.

Results and Discussion

Identification of the strain KNUA012

As shown in Fig. 1, the cyanobacterium was filamentous with straight trichomes composed of cylindrical cells. Cells were 1-2 μm wide and 20-50 μm long and not attenuated towards ends. End cells were slightly rounded and gas vacuoles were observed near the cross walls and sometimes distributed within the cells. Overall, strain KNUA012's morphological characteristics suggested that this isolate belonged to members of the genus *Limnothrix*.

Molecular characterization inferred from sequence analysis of the genes for 16S rRNA also indicated that the isolate belonged to the *Limnothrix* group (Fig. 2). Therefore, the isolate was tentatively identified as *Limnothrix* sp. KNUA
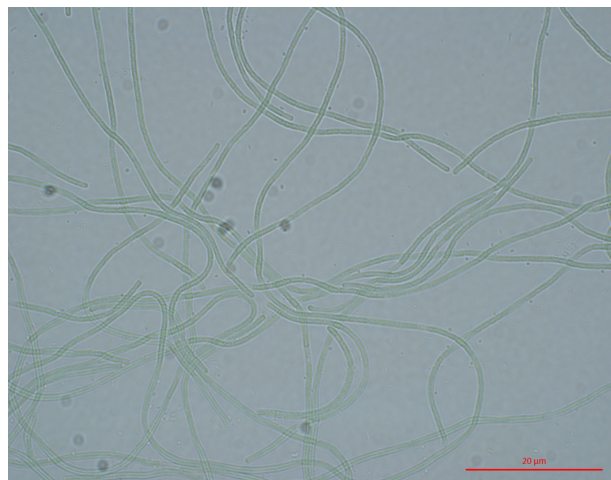

Fig. 1. Light microscopy of *Limnothrix* sp. KNUA012 (scale bar represents 20 μm).

012. However, the PC-IGS region sequence comparison revealed that uncultured cyanobacterium clone DC07PC-38 (HM020449) was the closest match for strain KNUA012 (Table 1). The 16S-23S ITS sequence analysis showed that the closest sequence match was *Geitlerinema unigranulatum* BCCUSP 94 (KJ735459) with a query coverage of only 82%. The *rbc*LX region sequence comparison also showed that uncultured *Limnothrix planktonica* KLL-C001 clone (KP 698043) was the closest match, but again the coverage was only 23%. This may be due to the lack of sequence data in GenBank for *Limnothrix* PC-IGS, 16S-23 ITS, and *rbc*LX genes, so no identification could be made with these data.

Table 2. List of the primers designed and used in this study

| Name	Sequence (5′ – 3′)	Length (bp)	Gene
alr5284F (forward)	ATG TTT GGT CTA ATT GGA CA	20	Partial acyl-ACP reductase
Lim_red_4R (reverse)	ATG CTC AAC AAT GCC ACC	18	Partial acyl-ACP reductase
ADDP1F (forward)	GTN ATH GAR GGN GA	14	Partial aldehyde decarbonylase
ADDP6R (reverse)	AAY TTY GGH GA	11	Partial aldehyde decarbonylase
KNUA012_RED_F (forward)	CCG TCT AGA CTG TCT CGG TT	20	Full acyl-ACP reductase
KNUA012_RED_R (reverse)	GGC TGA GTC CAT AGC CCT AG	20	Full acyl-ACP reductase
KNUA012_aldehyde_decarbonylase_ F (forward)	GTT TTC CGA CCG TTA CCC AC	20	Full aldehyde decarbonylase
KNUA012_aldehyde_decarbonylase_ R (reverse)	ATG TTG GCT GCA AAT GGG TT	20	Full aldehyde decarbonylase
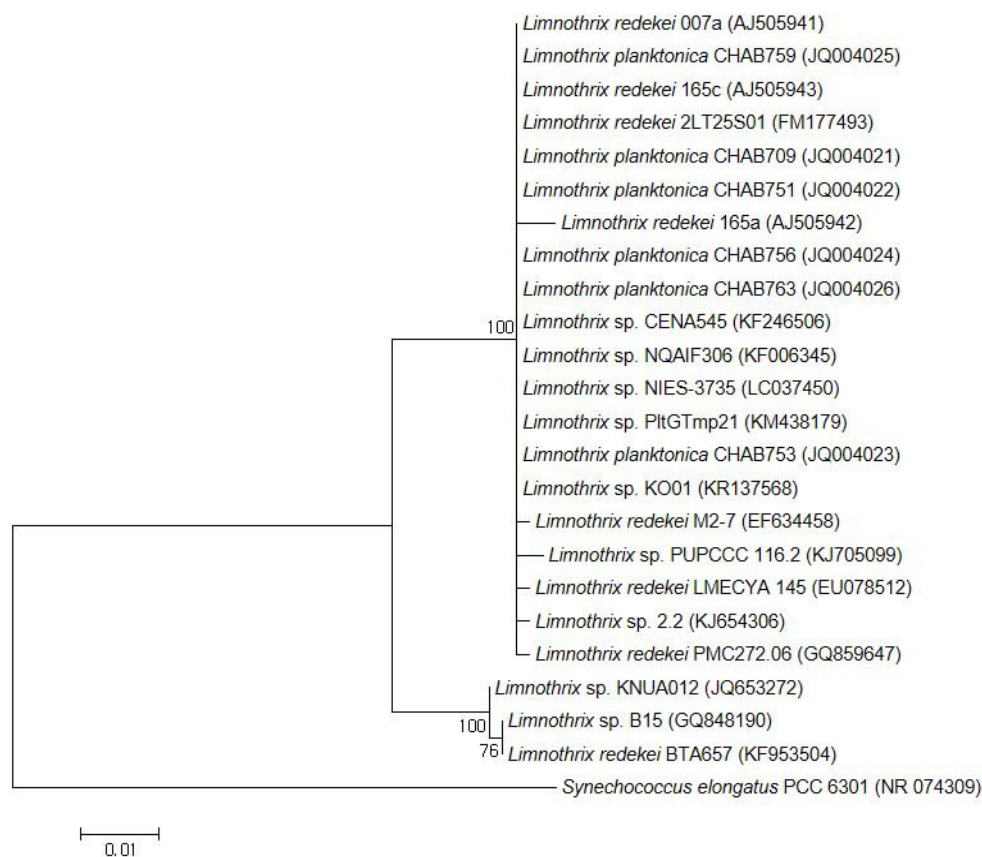
Fig. 2. The phylogenetic relationship of strain KNUA012 and its closely related species inferred from the 16S rRNA sequence data. The tree was generated by the maximum likelihood method with 1,000 bootstrap replicates. The scale bar represents a 1% difference in nucleotides sequences.

Table 3. Growth of strain KNUA012 at various temperatures

Temperature (°C)	5	10	15	20	25
OD_{750}	0.0	0.0	0.2	1.4	1.4

Optimal growth temperature

As shown in Table 3, strain KNUA012's maximal growth was obtained at ambient temperatures (20 and 25°C). However, no growth was observed at lower temperatures (5 and 10°C).

GC/MS analysis of strain KNUA012

It was found that strain KNUA012 was able to autotrophically synthesize alkanes (C_nH_{2n+2}) such as pentadecane and heptadecane. Since $C_{15}H_{32}$ and $C_{17}H_{36}$ are 15- and 17-carbon alkane hydrocarbons known as the major components of petrodiesel [14], this cyanobacterium-derived alkanes can be directly used as a biodiesel component without having to convert triglycerides into liquid hydrocarbons. In addition, other algal biodiesel constituents, $C_{14:1}$, $C_{16:0}$, $C_{16:1}$ were also produced by strain KNUA012. The GC/MS results are summarized in Table 4.

Table 4. Lipid profile of strain KNUA012

Component	Content (%)	Yield (mg g^{-1})
Lauric acid ($C_{12:0}$)	0.7±0.0	0.5
Myristic acid ($C_{14:0}$)	12.7±0.9	9.9
Myristoleic acid ($C_{14:1}$)	23.8±0.6	18.5
Pentadecane ($C_{15}H_{32}$)	3.0±0.7	2.3
Palmitic acid ($C_{16:0}$)	20.6±0.9	16.0
Palmitoleic acid ($C_{16:1}$)	30.0±1.9	23.2
8-heptadecene ($C_{17}H_{34}$)	0.7±0.1	0.6
Heptadecane ($C_{17}H_{36}$)	0.3±0.1	0.2
Stearic acid ($C_{18:0}$)	0.2±0.0	0.2
Oleic acid ($C_{18:1}$)	8.0±0.5	6.2

Biomass properties

In proximate analysis by TGA, the moisture content (MC) is determined by the mass loss before 110°C under N_2 atmosphere, the volatile matter (VM) refers to the mass loss between 110 – 900°C under N_2 as a result of thermal decom-
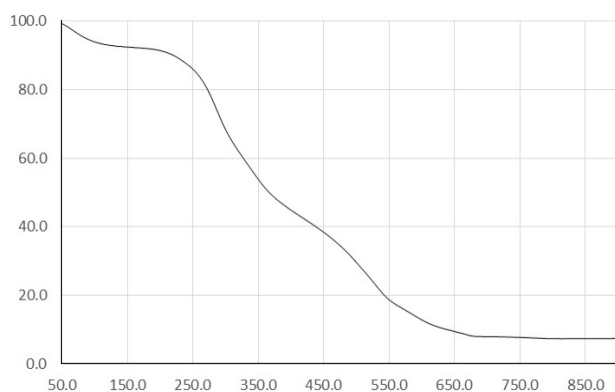
Fig. 3. TGA profiles of *Limnothrix* sp. KNUA012. The mass change in percentage is on the y-axis and temperature (°C) is on the x-axis.

Table 5. Proximate and ultimate analysis results of *Limnothrix* sp. KNUA012

Material component	Proximate analysis (wt%)	Elemental composition	Ultimate analysis (wt%)
MC	6.6	C	47.4
VM	86.0	H	6.7
FC + ash	7.4	N	7.3
		S	0.6
		HHV (MJ kg^{-1})	19.8

position, and the remaining mass represents fixed carbon (FC) and ash [2]. The moisture, VM, and FC and ash contents of strain KNUA012 were 6.6%, 86.0%, and 7.4%, respectively. The VM is defined as the part of solid fuel that is driven-off as a gas by heating and typical biomass generally has a VM content of up to 80%(crop residue: 63-80%; wood: 72-78%). The VM content of the cyanobacteria used in this study was higher than those of wood-based biomass feedstocks.

The HHV was also calculated to understand the potential of cyanobacterial biomass as a biofuel feedstock (Table 5). The result showed that the HHV was within the range of the terrestrial energy crops (17.0-20.0 MJ kg^{-1}) [22]. Given the higher photosynthetic efficiency and biomass productivity of microalgae [24], strain KNUA012 holds promise as a potential source for biomass feedstocks over crop plants. As high carbon content is a desirable property for fuel, if the higher concentration of CO_2 in the medium is available, the higher HHV are possible.

Strain KNUA012 was also able to autotrophically produce 21.4 mg g^{-1} PC. Because PC occurs as the major phycobiliprotein in cyanobacteria, it has been extracted from cyanobacteria such as *Spirulina platensis* and widely used in cosmetic and food industries. Recent studies have demonstrated the hepatoprotective, anti-inflammatory, and antioxidant properties of PC [18, 27]. This makes the isolate also desirable for commercial applications in the pharmaceutical industry.

Alkane biosynthesis genes

Partial sequences of an acyl-ACP reductase and an aldehyde decarbonylase in *Limnothrix* sp. KNUA012 were successfully obtained by the primer sets, alr5284F and Lim_red_4R, and ADDP1F and ADDP6R, respectively (Table 2). The downstream and upstream regions of the known sequences were further obtained using genome walking-PCR. The translated amino acid sequence of the hypothetical acyl-ACP reductase in *Limnothrix* sp. KNUA012 was most closely related to the long-chain fatty acyl-ACP reductase (WP_035997292) [*Leptolyngbya* sp. JSC-1] with 73% identity. In case of the hypothetical aldehyde decarbonylase, the closest match turned out to be the long-chain fatty aldehyde decarbonylase (WP_017659189) [*Geitlerinema* sp. PCC 7105] with an identity of 73%. The results are presented in Table 6.

The alkane biosynthesis pathway in cyanobacteria was first described by Schirmer *et al.* [25]. They demonstrated that fatty acyl-ACP was reduced to fatty aldehyde by a fatty acyl-ACP reductase and then converted into alkane by a fatty aldehyde decarbonylase, and it was found that both genes were required for alkane biosynthesis in cyanobacteria. Therefore, it seemed that these two hypothetical proteins in *Limnothrix* sp. KNUA012 may have resulted in the

Table 6. Acyl-ACP reductase and aldehyde decarbonylase genes in *Limnothrix* sp. KNUA012

Gene	The translated amino acid (Accession No. / length)	Closest match (Accession No. / % ID)
Acyl-ACP reductase	hypothetical acyl-ACP reductase (KU341740 / 369 amino acids)	Long-chain fatty acyl-ACP reductase [*Leptolyngbya* sp. JSC-1] (WP_035997292 / 73%)
Aldehyde decarbonylase	hypothetical aldehyde decarbonylase (KU341741 / 234 amino acids)	Long-chain fatty aldehyde decarbonylase [*Geitlerinema* sp. PCC 7105] (WP_017659189 / 73%)

production of odd-chain alkanes ($C_{15}H_{32}$ and $C_{17}H_{36}$). However, it should be stated that the alkane yields from wild type cyanobacteria are still insufficient to compete with petroleum derived fuels [4, 10] and the over expression of alkane synthesis pathways is necessary [33, 34]. Future work could be required to see whether the gene set from strain KNUA012 would confer alkane production to heterologous hosts such as *E. coli* or yeast.

In conclusion, this Korean indigenous cyanobacterium could serve as potential biological resource to produce various compounds of biochemical interest. The real potential of the isolate described in this paper should be evaluated through further cultivation studies at molecular, laboratory, and field scales.

Acknowledgements

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초록 : 토착 남세균 림노트릭스 속 KNUA012 균주의 바이오연료 원료로서의 특성 연구

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사상체 토착 남세균을 경상남도 합천군 합천호의 수화시료로부터 무균적으로 분리하였으며, 형태적 · 분자적 동정 결과 림노트릭스 속에 속하는 것으로 밝혀졌다. 따라서, 본 남세균 균주는 림노트릭스 속 KNUA012 균주로 명명하였으며, 분리균주의 최적생장 온도는 섭씨 25도였다. 지질성분 분석 결과, 에스테르 교환반응을 거치지 않고 직접 연료로 사용할 수 있는 펜타데칸($C_{15}H_{32}$)과 헵타데칸($C_{17}H_{36}$)과 같은 알칸들이 본 균주에 의해 광독립 영양적으로 생합성 된다는 것이 밝혀졌다. 또한 알칸 생합성에 관여하는 유전자들이 본 남세균 내에 존재하는 것을 발견하였다. 일반적인 미세조류 바이오디젤 구성성분으로 알려진 미리스트올레산($C_{14:1}$), 팔미트산($C_{16:0}$) 및 팔미톨레산($C_{16:1}$) 역시 KNUA012 균주에 의해 주요 지방산 성분으로서 생산되는 것으로 확인되었다. 근사분석 결과 KNUA012 균주의 휘발물질 함량은 86.0%였으며, 원소분석 결과 고위발열량은 19.8 MJ kg^{-1}으로 나타났다. 또한, 본 분리균주는 고부가가치 항산화물질로 알려져 있는 피코시아닌을 광독립영양적으로 21.4 mg g^{-1}의 농도로 생산할 수 있는 것을 확인하였다. 따라서, 본 연구결과는 KNUA012 균주가 미세조류 기반 바이오연료와 바이오매스 원료의 경제적인 생산에 있어 이상적인 자원이 될 수 있음을 제시하였다.