Biochemical Composition of a Korean Domestic Microalga Chlorella vulgaris KNUA027

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Recently, photosynthetic microalgae have gained particular interest as a new source for industrially important biomolecules because they are able to convert carbon dioxide (CO₂) to various types of products such as carbohydrates, lipids, and proteins with minimal growth requirements [6, 29]. In particular, microalgae are now considered as one of the most attractive candidates for biofuel and polysaturated fatty acid (PUFA) production due to their higher photosynthetic efficiency and oil yield compared to terrestrial crops [16, 20, 37]. In this study, a Korean indigenous microalga, Chlorella vulgaris KNUA027 was isolated and identified, and its potential as biofuel and PUFA feedstock was investigated.

A unicellular green alga, Chlorella vulgaris KNUA027, was isolated from the roots of Panax ginseng seedlings and its biotechnological potential was investigated. The results of GC/MS analysis showed that C. vulgaris KNUA027 was rich in nutritionally important polysaturated fatty acids (PUFAs) such as alpha-linolenic acid (C₁₈:₃ ω₃, 45.8%, 50.8 mg/g) and hexadecatrienoic acid (C₁₆:₃ ω₃, 11.8%, 13.1 mg/g). Therefore, this Korean indigenous microalga may have potential as a source of omega-3 PUFAs. It was also found that the saturated palmitic acid (C₁₆:₀, 37.1%, 41.2 mg/g), which is suitable for biodiesel production, was one of the major fatty acids produced by strain KNUA027. The proximate analysis showed that the volatile matter content was 88.5%, and the ultimate analysis indicated that the higher heating value was 19.8 MJ/kg. Therefore, the results from this research with C. vulgaris KNUA027 may provide the basis for the production of microalgae-based biofuels and biomass feedstock.

Keywords: Biofuel feedstock, Chlorella vulgaris, microalga, PUFA

A unicellular green alga, Chlorella vulgaris KNUA027, was isolated from the roots of Panax ginseng seedlings and its biotechnological potential was investigated. The results of GC/MS analysis showed that C. vulgaris KNUA027 was rich in nutritionally important polysaturated fatty acids (PUFAs) such as alpha-linolenic acid (C₁₈:₃ ω₃, 45.8%, 50.8 mg/g) and hexadecatrienoic acid (C₁₆:₃ ω₃, 11.8%, 13.1 mg/g). Therefore, this Korean indigenous microalga may have potential as a source of omega-3 PUFAs. It was also found that the saturated palmitic acid (C₁₆:₀, 37.1%, 41.2 mg/g), which is suitable for biodiesel production, was one of the major fatty acids produced by strain KNUA027. The proximate analysis showed that the volatile matter content was 88.5%, and the ultimate analysis indicated that the higher heating value was 19.8 MJ/kg. Therefore, the results from this research with C. vulgaris KNUA027 may provide the basis for the production of microalgae-based biofuels and biomass feedstock.

Keywords: Biofuel feedstock, Chlorella vulgaris, microalga, PUFA

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transferred to fresh BG-11 plates to obtain an axenic algal culture.

For morphological identification, live cells were harvested, suspended in sterile distilled water, and inspected at ×1,000 magnification on a Nikon Eclipse E100 Biological Microscope (Japan). For molecular analysis, genomic DNA was extracted using a DNeasy Plant Mini kit (Qiagen, Germany). The primer sets NS1/NS8 and ITS1/ITS4 [39] were used to amplify the 18S rRNA gene and internal transcribed spacer (ITS) region, respectively. The D1-D2 region of the large subunit rRNA gene of the isolate was amplified using the NL1 and NL4 primers [27]. The ITS2 rRNA secondary structure of strain KNUA027 was predicted in the ITS2 Database (http://its2.bioapps.biozentrum.uni-wuerzburg.de) [18, 40]. Phylogenetic analysis was performed with the ITS sequence of strain KNUA027 using the software package MEGA ver. 6.0 [38]. Its closely related Chlorella sequences were downloaded and aligned in the MEGA software, with the ClustalW tool. The best-fit nucleotide substitution model (T92) was selected using MEGA 6.0 based on the Bayesian information criterion. This model was used to build a maximum likelihood (ML) phylogenetic tree with 1,000 bootstrap replicates. Due to the highly conserved nature of rRNA, the plastid-encoded \( psaA \) (photosystem I P700 chlorophyll a apoprotein A1) and \( psbA \) (photosystem II reaction center protein D1) were also sequenced using primer sets, \( psaA130F-psaA1760R \) for \( psaA \) and \( psbAF1-psbAR2 \) for \( psbA \), respectively [42]. The DNA sequences obtained were submitted to the NCBI database and their accession numbers were listed in Table 2. Also, the strain obtained in this study was deposited in the Korean Collection for Type Cultures (KCTC) under the accession number KCTC 12965BP.

For biomass characterization, the isolate was inoculated into BG-11 medium in triplicate and incubated at 25°C for 20 days until the culture reached its late exponential phase. Cells were harvested by centrifugation at 3,220 \( g \) (Centrifuge 5810R, Eppendorf, Germany) and immediately freeze-dried. The lipids were then extracted using a modified version of the Bligh-Dyer method [41]. The fatty acid composition of the cultures was decided by GC/MS (Jeol JMS700 mass spectrometer equipped with an Agilent 6890N GC, Agilent Technologies, USA). Peak identification and compound assignment were performed based on electron impact mass spectrum and the National Institute of Standards and Technology mass spectral libraries [35] were used as reference databases.

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. [10]: \[ \text{HHV} = 3.55C^2 - 232C - 2,230H + 51.2C \times H + 131N + 20,600 \text{ (MJ/kg)} \]. Protein content was calculated

### Table 1. Composition of BG-11 medium.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO(_3)</td>
<td>1.5</td>
</tr>
<tr>
<td>CaCl(_2)-2H(_2)O</td>
<td>0.036</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.012</td>
</tr>
<tr>
<td>EDTA-Na(_2)-2H(_2)O</td>
<td>0.001</td>
</tr>
<tr>
<td>K(_2)HPO(_4)</td>
<td>0.04</td>
</tr>
<tr>
<td>MgSO(_4)-7H(_2)O</td>
<td>0.075</td>
</tr>
<tr>
<td>Na(_2)CO(_3)</td>
<td>0.02</td>
</tr>
<tr>
<td>Trace metal solution(^a)</td>
<td>1 ml/l</td>
</tr>
</tbody>
</table>

\(^a\)H\(_3\)BO\(_3\), 2.86 g/l; MnCl\(_2\)-4H\(_2\)O, 1.81 g/l; ZnSO\(_4\)-7H\(_2\)O, 0.222 g/l; Na\(_2\)MoO\(_4\)-2H\(_2\)O, 0.39 g/l; CuSO\(_4\)-5H\(_2\)O, 0.079 g/l; Co(NO\(_3\))\(_2\)-6H\(_2\)O, 0.049 g/l.

### Table 2. BLAST sequence alignment output using 5 different marker genes of *Chlorella vulgaris* KNUA027.

<table>
<thead>
<tr>
<th>Marker gene</th>
<th>Accession number</th>
<th>Length (bp)</th>
<th>Closest match (GenBank accession number)</th>
<th>Overlap (%)</th>
<th>Sequence similarity (%)</th>
<th>Taxonomic affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>KU306723</td>
<td>1,771</td>
<td><em>Chlorella vulgaris</em> CCAP 211/21A (KJ756823)</td>
<td>100</td>
<td>100</td>
<td><em>Chlorella vulgaris</em></td>
</tr>
<tr>
<td>ITS</td>
<td>KU306724</td>
<td>782</td>
<td><em>Chlorella vulgaris</em> CCAP 211/115 (FR865660)</td>
<td>100</td>
<td>99</td>
<td><em>Chlorella vulgaris</em></td>
</tr>
<tr>
<td>28S rRNA</td>
<td>KU306725</td>
<td>517</td>
<td><em>Chlorella vulgaris</em> LS 120 (KC912856)</td>
<td>100</td>
<td>100</td>
<td><em>Chlorella vulgaris</em></td>
</tr>
<tr>
<td>psaA</td>
<td>KX066372</td>
<td>1,611</td>
<td><em>Chlorella vulgaris</em> NIES-227 (AB260919)</td>
<td>99</td>
<td>99</td>
<td><em>Chlorella vulgaris</em></td>
</tr>
<tr>
<td>psbA</td>
<td>KX066373</td>
<td>998</td>
<td><em>Chlorella vulgaris</em> C-27 (AB001684)</td>
<td>100</td>
<td>99</td>
<td><em>Chlorella vulgaris</em></td>
</tr>
</tbody>
</table>
from the N content in the ultimate analysis by using the conversion factor (× 6.25) [23]. Proximate analysis was carried out on a DTG-60A thermal analyzer (Shimadzu, Japan). Platinum pans were used to contain 30 mg of α-alumina (α-Al₂O₃) powder (Shimadzu, Japan) as a reference material and approximately 10 mg of each sample, respectively. Nitrogen (> 99.999%, N₂) was supplied as the carrier gas at a rate of 25 ml/min to protect the microalgae powder from oxidation. Samples were heated from 50 to 900°C at a rate of 10°C/min. Thermogravimetric analysis (TGA) data were analyzed by ta60 Ver. 2.21 software (Shimadzu, Japan).

Strain KNUA027 had common features of the genus *Chlorella*. The algal cells were solitary, non-motile, and

Fig. 1. Light microscopy of *Chlorella vulgaris* KNUA027 at ×1,000 magnification on a Nikon Eclipse E100 Biological Microscope (Japan).

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Fig. 2. The phylogenetic relationship of strain KNUA027 and its closely related species inferred from the ITS sequence data.
Domestic \textit{C. vulgaris} as Biochemical Feedstock

round to slightly ellipsoid in shape. The microorganism had a predominant cup-shaped chloroplast and its sizes ranged from approximately 1–3 µm in diameter (Fig. 1). Molecular characterization inferred from sequence analyses of the genes for 18S rRNA, 28S rRNA, the ITS region, \textit{psa}A, and \textit{psb}A showed that the isolate belonged to the \textit{C. vulgaris} group (Fig. 2, Table 1). Furthermore, strain KNUA027 exhibited a G-C pairing on the top of helix III (Fig. 3). These key compensatory base changes (CBCs) in the ITS2 secondary structure also confirmed that strain KNUA027 belonged to the genus \textit{Chlorella} [15]. Therefore, the isolate was identified as \textit{C. vulgaris} strain KNUA027.

The fatty acid profile of \textit{C. vulgaris} KNUA027 is summarized in Table 3. Analysis of the cellular fatty acid composition of strain KNUA027 revealed that \(\alpha\)-linolenic acid (ALA, 45.8%, 50.8 mg/g), palmitic acid (37.1%, 41.2 mg/g), and hexadecatrienoic acid (HTA, 11.8%, 13.1 mg/g) were the major fatty acids. Numerous studies have demonstrated that ALA and HTA have many beneficial health effects [25] and various commercial omega-3 products are available worldwide [28]. As omega-3 PUFAs are primarily derived from refined fish oils, this isolate may have the potential to be used as an alternative to fish-based sources. The 16-carbon saturated palmitic acid suitable for biodiesel production was also autotrophically biosynthesized by strain KNUA027 as one of the major fatty acids. Recent studies on the biodiesel production by \textit{Chlorella vulgaris} have demonstrated that palmitic acid (C\textsubscript{16:0}), linolenic acid (C\textsubscript{18:2}), linoleic acid (C\textsubscript{18:2}), and oleic acid (C\textsubscript{18:1}) were the main fatty acids regardless of culture medium and \textit{C. vulgaris}

\begin{table}[h]
\centering
\caption{Fatty acid profile of \textit{Chlorella vulgaris} KNUA027.}
\begin{tabular}{lcc}
\hline
Component & Content (\%) & Yield (mg/g DW) \\
\hline
8-heptadecene (C\textsubscript{17}H\textsubscript{34}) & 1.4 & 1.6 \\
Heptadecane (C\textsubscript{17}H\textsubscript{36}) & 0.7 & 0.8 \\
9,12-cis-hexadecadienoic acid (C\textsubscript{16:2} ω\textsubscript{4}) & 0.6 & 0.7 \\
Hexadecatrienoic acid (C\textsubscript{16:3} ω\textsubscript{3}) & 11.8 & 13.1 \\
Palmitic acid (C\textsubscript{16:0}) & 37.1 & 41.2 \\
Linoleic acid (C\textsubscript{18:2} ω\textsubscript{6}) & 2.7 & 3.0 \\
\(\alpha\)-Linolenic acid (C\textsubscript{18:3} ω\textsubscript{3}) & 45.8 & 50.8 \\
\hline
\end{tabular}
\end{table}

Fig. 3. ITS2 secondary structure for \textit{Chlorella vulgaris} KNUA027. The key CBCs at the tip of helix III are indicated in a box.
strain (Table 4). Likewise, palmitic acid (37.1%) was one of the most abundant fatty acids in strain KNUA027. However, the high content of ALA (45.8%) makes the isolate an interesting candidate for further in-depth study involving omega-3 production. In addition, *C. vulgaris* KNUA027 was reported to produce a trace amount of heptadecane (0.7%, 0.8 mg/g). As heptadecane is a 17-carbon alkane hydrocarbon known as one of the major components of petrodiesel [19], this microalga-derived alkane can be directly used as a biodiesel component without having to convert triglycerides into liquid hydrocarbons.

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 decomposition, and the remaining mass represents fixed carbon (FC) and ash [5]. The moisture, VM, and FC and ash contents of strain KNUA027 were 5.0%, 88.5%, and 6.5%, respectively (Fig. 4). 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 microalga used in this study was higher than the range of wood-based biomass feedstocks.

The HHV was also calculated to understand the potential of algal biomass as a biofuel feedstock (Table 5). The results showed that the HHV was within the range of the terrestrial energy crops (17.0−20.0 MJ/kg) [32]. A number of previous studies have been demonstrated on the HHVs of *Chlorella* strains under different autotrophic growth conditions and the biomass samples were characterized in the range of around 20.0−30.0 MJ/kg.

Table 4. Major fatty acids produced by *Chlorella vulgaris* strains from previous studies.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Major fatty acid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTEX 259</td>
<td>BBM</td>
<td>Oleic acid (C$<em>{18}$, 61.0%), palmitic acid (C$</em>{16}$, 24.6%)</td>
<td>[1]</td>
</tr>
<tr>
<td>Not specified</td>
<td>SWM$^a$</td>
<td>Linolenic acid (C$<em>{18}$, 22.1%), oleic acid (C$</em>{18}$, 19.6%)</td>
<td>[2]</td>
</tr>
<tr>
<td>CCAP 211/11</td>
<td>MBL</td>
<td>Linoleic acid (C$<em>{18}$, 24.6%), palmitic acid (C$</em>{16}$, 23.7%)</td>
<td>[3]</td>
</tr>
<tr>
<td>Not specified</td>
<td>BBM</td>
<td>Palmitic acid (C$<em>{16}$, 19.8%), linoleic acid (C$</em>{18}$, 17.7%)</td>
<td>[4]</td>
</tr>
<tr>
<td>TISTR 8261$^b$</td>
<td>Chu13</td>
<td>Palmitic acid (C$<em>{16}$, 41.4%), oleic acid (C$</em>{18}$, 27.8%)</td>
<td>[7]</td>
</tr>
<tr>
<td>Not specified</td>
<td>BG-11</td>
<td>Linolenic acid (C$<em>{18}$, 28.2%), linoleic acid (C$</em>{18}$, 18.2%)</td>
<td>[8]</td>
</tr>
<tr>
<td>CCAP 211</td>
<td>BBM</td>
<td>Palmitic acid (C$<em>{16}$, 63.0%), linolenic acid (C$</em>{18}$, 13.0%)</td>
<td>[9]</td>
</tr>
<tr>
<td>CCAP 211</td>
<td>BBM</td>
<td>Palmitic acid (C$<em>{16}$, 39.2%), linolenic acid (C$</em>{18}$, 20.4%)</td>
<td>[11]</td>
</tr>
<tr>
<td>INETI 58</td>
<td>CM$^c$</td>
<td>Palmitic acid (C$<em>{16}$, 25.1%), linolenic acid (C$</em>{18}$, 19.1%)</td>
<td>[12]</td>
</tr>
<tr>
<td>Not specified</td>
<td>BG-11</td>
<td>Palmitic acid (C$<em>{16}$, 53.0%), linolenic acid (C$</em>{18}$, 8.9%)</td>
<td>[13]</td>
</tr>
<tr>
<td>UTEX 2714</td>
<td>OCM$^d$</td>
<td>Palmitic acid (C$<em>{16}$, 32.0%), oleic acid (C$</em>{18}$, 18.0%)</td>
<td>[14]</td>
</tr>
<tr>
<td>UTEX 395</td>
<td>BBM</td>
<td>Oleic acid (C$<em>{18}$, 16.8%), linolenic acid (C$</em>{18}$, 9.3%)</td>
<td>[21]</td>
</tr>
<tr>
<td>Not specified</td>
<td>N11</td>
<td>Palmitic acid (C$<em>{16}$, 62.4%), stearic acid (C$</em>{18}$, 19.5%)</td>
<td>[22]</td>
</tr>
<tr>
<td>Not specified</td>
<td>Conway</td>
<td>Palmitic acid (C$<em>{16}$, 29.1%), linoleic acid (C$</em>{18}$, 24.6%)</td>
<td>[24]</td>
</tr>
<tr>
<td>Not specified</td>
<td>MBM</td>
<td>Palmitic acid (C$<em>{16}$, 22.9%), oleic acid (C$</em>{18}$, 21.5%)</td>
<td>[26]</td>
</tr>
<tr>
<td>211/11B</td>
<td>BBM</td>
<td>Linoleic acid (C$<em>{18}$, 22.1%), palmitic acid (C$</em>{16}$, 19.8%)</td>
<td>[36]</td>
</tr>
<tr>
<td>CCTCC M 209256</td>
<td>SSM$^e$</td>
<td>Oleic acid (C$<em>{18}$, 45.4%), palmitoleic acid (C$</em>{16}$, 23.3%)</td>
<td>[43]</td>
</tr>
</tbody>
</table>

$^a$Synthetic wastewater medium; $^b$Co-culture with *Rhodotorula glutinis*; $^c$Chlorella medium; $^d$Optimized culture medium; $^e$Synthetic seawater medium.

Fig. 4. TGA profile of *Chlorella vulgaris* KNUA027. The mass change in percentage is on the y-axis and temperature (°C) is on the x-axis [5].
Table 5. Sample component and elemental composition of Chlorella vulgaris KNUA027.

<table>
<thead>
<tr>
<th>Component</th>
<th>Proximate analysis (wt%)</th>
<th>Elemental composition</th>
<th>Ultimate analysis (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC</td>
<td>5.0</td>
<td>C</td>
<td>47.4</td>
</tr>
<tr>
<td>VM</td>
<td>88.5</td>
<td>H</td>
<td>6.7</td>
</tr>
<tr>
<td>FC + Ash</td>
<td>6.5</td>
<td>N</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>0.6</td>
</tr>
<tr>
<td>HHV</td>
<td></td>
<td></td>
<td>19.8</td>
</tr>
</tbody>
</table>

Moisture content; Volatile matter; Fixed carbon; Higher heating value.

[17, 31, 34]. However, these HHV results cannot be directly compared with our results because of the different culture conditions. Given the higher photosynthetic efficiency and biomass productivity [33], strain KNUA027 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 CO₂ in the medium is available, the higher HHV are possible. In addition, the biomass may also serve as an excellent animal feed because of its high protein content (45.7%).

In conclusion, this Korean indigenous microalga, Chlorella vulgaris KNUA027 could serve as potential biological resource to produce 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.

Acknowledgments

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국문초록

한국 토착 미세조류 클로렐라 불가리스 KNUA027 군주의 생화학적 조성

국립해양생물자원관 해양식물팀
경북대학교 신바이오소재연구소
경북대학교 에너지과학과
경북대학교 전단복합 생명과학인력 양성사업단
동신대학교 생물자원산업지원센터
동신대학교 올릉도·독도연구소

인삼 유묘 쿠파 주변에서 자라고 있는 단쇄 녹색 조류, 클로렐라 불가리스 KNUA027을 순수분리한 후 본 분리군주의 생물공학적 활용 가능성을 위해 조사를 실시하였다. 가스크로마토그래프/질량분석기를 이용한 분석 결과, 본 군주에는 영양학적으로 중요한 알파 리놀렌산(C18:3 ω3, 45.8%, 50.8 mg/g) 및 헥사데카트리엔산(C16:3 ω3, 11.8%, 13.1 mg/g)과 같은 다가불포화지방산이 풍부한 것으로 밝혀졌다. 따라서, 본 국내 토착 미세조류는 잠재적인 오메가-3 다가불포화지방산 원료가 될 수 있다고 사료된다. 또한, 바이오디젤 생산에 적합한 것으로 알려진 캐피토산(C16:0, 37.1%, 41.2 mg/g) 역시 본 군주에 의해 주요 지방산 성분으로 생합성되는 것으로 확인되었다. 근사분석 결과 KNUA027 군주의 화발성물질 함량은 88.5%였으며, 원소분석 결과 고위발열량은 19.8 MJ/kg으로 나타났다. 본 KNUA027 군주를 이용한 연구결과는 미세조류 기반 바이오연료와 바이오매스 생산을 위한 기초자료 역할을 할 수 있을 것으로 기대된다.