Biomass and Bioenergy 85 (2016) 300–305

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: http://www.elsevier.com/locate/biombioe

Research paper

Chlorella sorokiniana HS1, a novel freshwater green algal strain, grows and hyperaccumulates lipid droplets in seawater salinity

Byung-Hyuk Kim [a, 1], Rishiram Ramanan [a, 1], Zion Kang [a, b], Dae-Hyun Cho [a], Hee-Mock Oh [c, d], Hee-Sik Kim [a, d, *]

[a] Sustainable Bioresource Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, 305-806, Republic of Korea
[b] Advanced Biomass R&D Center, Korea Advanced Institute of Science & Technology (KAIST), 291 Daehakno, Yuseong-gu, Daejeon 305-701, Republic of Korea
[c] Bioenergy and Biochemical Research Center, KRIBB, Yuseong-gu, Daejeon 305-806, Republic of Korea
[d] Major of Green Chemistry and Environmental Biotechnology, University of Science & Technology (UST), Daejeon 305-806, Republic of Korea

ARTICLE INFO

Article history:
Received 12 March 2015
Received in revised form
16 December 2015
Accepted 22 December 2015
Available online xxx

Keywords:
Chlorella
Salt stress
Green algae
Lipid droplets
Biodiesel
Commercialization

ABSTRACT

Salinity is a major abiotic stress for terrestrial plants and freshwater microalgae alike. The most notable physiological response of microalgae like *Dunaliella* to salinity stress is reduced photosynthesis and production of carotenoids. Here, we report isolation and characterization of a novel freshwater microalgal strain, *Chlorella sorokiniana* HS1, which grows and hyperaccumulates lipid droplets (LD) in hypersaline conditions greater than seawater salinity. Other freshwater *Chlorella* strains tested neither grew nor possessed high LD levels in high salt concentration. *C. sorokiniana* HS1 displayed increase in cell size (200%) and LD, resulting in increased biomass and lipid productivity, respectively, with altered but favourable fatty acid methyl ester composition under salinity stress. Experimental analyses reveal definitive shift from proteins and starch to LD synthesis as well as chlorophyll degradation, response analogous to nitrogen starvation. Acute salt stress (<6 h) in seawater salinity or above resulted in instant accumulation of LD. *C. sorokiniana* HS1 allows for two phase cultivation, growth phase in freshwater and stress induction phase in seawater for biodiesel production. This strain would therefore significantly reduce costs and production constraints associated with stress induction.

1. Introduction

Microalgae provide several advantages for biodiesel production over oil crops as they are not dependent on fertile land, offer higher productivities and can use wastewater [1]. Algae also possess certain disadvantages including high water footprint, high nutrient input, low cell density and harvesting issues, prone to predation and competition, and more importantly, accumulate lipids to desired levels only under stress conditions like nutrient starvation [2–4]. And these conditions, especially nutrient starvation, is difficult to achieve in large scale systems, eventually increasing cost of production and water footprint, rendering microalgal biodiesel unviable. Considering this critical limitation of nutrient starvation for large scale application, it is important to continually explore other lipid accumulating conditions and organisms more suitable for large scale cultivation [5].

Salt stress is proven to be easier to achieve in mass cultivation. However, the major known response of microalgae towards salt stress, as in the case of *Dunaliella* and *Haematococcus* is the production of carotenoids and glycerol [6]. Since induction of salt stress is demonstrated at large scale, it would be one of the easiest stress conditions to emulate for biodiesel applications, provided a strain which naturally accumulates lipids under salinity stress is found [7]. *Chlorella* is considered a model alga for biodiesel application because of its robustness towards environmental stress and interspecies competition, and ability to grow in wastewater. Thus, *Chlorella* is the only green alga with biodiesel production potential to be produced by over 70 companies for high-value products on a commercial scale [6]. Green alga *Chlorella sorokiniana*, the most promising of *Chlorella* strains for biodiesel applications, is common in freshwater and is known to be inhibited in 0.035 g L^{-1} NaCl concentration, but is known to be heat, pH and light tolerant [8,9].

* Corresponding author. Sustainable Bioresource Research Center, KRIBB, Daejeon 305-806, Republic of Korea.
E-mail address: hkim@kribb.re.kr (H.-S. Kim).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.biombioe.2015.12.026
0961-9534/

It is also a reliable source of essential fatty acids that are required for many important biochemical functions [10] and reproduces at an extremely fast rate, renewing into four new cells in every 9–17 h [11,12]. The genome of this promising alga is expected to be published shortly, which might potentially accelerate the efforts of biodiesel production.

This study reports the isolation of a novel *C. sorokiniana* HS1 strain which grows even at 60 g L^{-1} NaCl, which is much higher than seawater salinity. Biochemical, physiological and intracellular structural changes examined under seawater salinity show that this strain can be scaled up for biodiesel production. Moreover, this strain can serve as a model organism to study stress response through carbon flux diversion from starch and protein towards LDs.

2. Material and methods

2.1. C. sorokiniana isolation and culture condition

C. sorokiniana HS1 was isolated from swine wastewater by serial dilution and cultured in 125 cm^3 Erlenmeyer flasks in BG11 medium at 28 °C for 15 d with a light intensity of 50 $\mu Em^{-2}s^{-1}$. The algal strain was cultivated in BG11 medium containing (g L^{-1}): $K_2HPO_4 \cdot 3H_2O$, 0.04; $MgSO_4 \cdot 7H_2O$, 0.075; $CaCl_2 \cdot 2H_2O$, 0.036; citric acid, 0.006; ferric ammonium citrate, 0.006; EDTA, 0.001; $NaNO_3$, 1.5; Na_2CO_3, 0.02; trace metal mix A5, 1.0 cm^3. Trace metal mix A5 solution consisted of (g L^{-1}) H_3BO_3, 2.86; $MnCl_2 \cdot 4H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.222; $NaMoO_4 \cdot 2H_2O$, 0.39; $CuSO_4 \cdot 5H_2O$, 0.079; and $CoCl_2 \cdot 6H_2O$, 0.05. *C. sorokiniana* HS1 was able to grow in all the tested concentrations of sodium chloride (0–60 g L^{-1}). The salinity of the culture media used was 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 g L^{-1} NaCl. For salt shock condition, the strain was grown in BG11 media until late exponential phase, centrifuged and the pellet was transferred to fresh BG11 media with different salt concentrations.

2.2. Cultivation of several Chlorella sp. in salt stress

To understand if resistance to salinity stress was unique to this strain or prevalent among *Chlorella* sp., six strains including *C. sorokiniana* HS1 (C1: *C. ellipsoidea* NIES 2150; C2: *C. protothecoides* UTEX 1806; C4: *C. regularis* UTEX 1807; AG: *C. sorokiniana* AG20740; OW: *Chlorella vulgaris* OW01; HS: *C. sorokiniana* HS1) were grown in BG11 medium with different NaCl concentrations (0, 10, 20, 30, 40 and 50 g L^{-1}). Optical density was measured using a microplate reader (Tecan, Switzerland) at 680 nm every alternate day till 10 d and specific growth rate was calculated. After 10 d of growth, the strains were stained with Nile red and fluorescence of LD was measured using a microplate fluorescence reader (BioTek, USA) [13].

2.3. DNA extraction, 18S rRNA gene amplification and phylogenetic analysis

The microalgal biomass was washed twice with TE buffer (Tris 10 mmol m^{-3}, EDTA 1 mmol m^{-3}, pH 8.0) after centrifugation at 4800 × g for 5 min, resuspended in 1.5 cm^3 distilled water and centrifuged at 10,000 × g for 3 min at room temperature. DNA extractions were carried out in accordance with modified eukaryotic microalgal nucleic acids extraction (EMNE) method [14].

The algal specific 18S rRNA gene amplification was performed using primers 18SF (5′- CCT GGT TGA TCC TGC CAG -3′), 18SR (5′- TTG ATC CTT CTG CAG GTT CA -3′) to obtain an amplicon of 1,740 base pair [15]. Rest of the protocol was performed as described elsewhere [14]. Phylogenetic analysis was performed by aligning the 18S rRNA sequences obtained with the reference sequences and constructing the tree using maximum likelihood method [16]. The sequences of 18S rRNA gene were aligned by 100 CLUSTAL X [17] and edited by BIOEDIT [18] with published sequences retrieved from NCBI database (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were reconstructed using the neighbour-joining [19], maximum-parsimony [20], and maximum-likelihood [21] algorithms in the MEGA 5 software [22] with bootstrap values based on 1000 replications [21]. The sequence was also submitted to NCBI (Accession Number DI373817).

2.4. Transmission electron Microcopy (TEM) imaging of C. sorokiniana HS1

C. sorokiniana HS1 cells grown at different salt concentrations (0, 10, 20 and 30 g L^{-1}) for 12 d were centrifuged (× 1000 g, 10 min) and the pellet was fixed in 25 g L^{-1} paraformaldehyde-glutaraldehyde mixture buffered with 0.1 mol m^{-3} phosphate (pH 7.2) for 2 h, post-fixed in 10 g L^{-1} osmium tetroxide in the same buffer for 1 h, dehydrated in graded ethanol and propylene oxide, and embedded in Epon-812. Ultra-thin sections, prepared using a Leica ULTRACUT E ultramicrotome (Leica, Illinois, USA), were stained with uranyl acetate and lead citrate and examined under a CM-20 electron microscope (Philips Electron Optics, Eindhoven, Netherlands).

2.5. Biochemical analyses

Lipid extraction, fatty acid methyl ester (FAME), and chlorophyll-*a* analysis were performed as mentioned in earlier studies [13,23]. Total protein analysis was performed by semi-micro Kjeldahl digestion method [24]. Starch level was determined with the Starch Assay Kit (Sigma–Aldrich, MO, USA) according to manufacturer's protocol.

3. Results and discussion

3.1. Identification of Chlorella sorokiniana HS1

The results of 18S rRNA gene sequence of *C. sorokiniana* HS1 indicate that the closest relative of strain HS1 is *C. sorokiniana* strain Prag A14, with over 99% similarity. A phylogenetic tree based on 18S rRNA gene sequences of HS1 and the species within genus *Chlorella* was constructed which further illustrates that the new strain HS1 is most closely related to *C. sorokiniana* (Fig. 1). On the basis of 18S rRNA gene sequence and morphological analysis, the isolate was identified as *C. sorokiniana* HS1.

3.2. Response to salinity stress

Since *C. sorokiniana* HS1 was isolated from swine wastewater, which has relatively high salinity levels, tolerance levels of this strain towards higher salt concentrations was tested. Surprisingly, the strain was able to grow in all the tested concentrations of NaCl (0–60 g L^{-1}). It has been earlier reported that *Chlorella* sp. can tolerate only mild salinity levels typically (<10 g L^{-1}) [8,9]. Therefore, five other *Chlorella* strains were grown in BG11 medium with different salt concentrations. Among the tested strains, *C. sorokiniana* HS1 showed consistent growth among all replicates and had high specific growth rate even at seawater salinity (Fig. 2 & Supplementary figure 1). Other strains including *C. sorokiniana* AG20740 did not show consistent growth in increasing salt concentrations (Supplementary table 1). These results demonstrated that this strain had an unique ability to grow under salt stress in spite of being a freshwater strain, as it showed the highest growth rate in BG11 (No supplemented NaCl, control). As stress conditions are known to induce either carotenoid