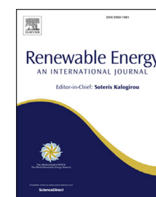
Renewable Energy 98 (2016) 72–77

Contents lists available at ScienceDirect

Renewable Energy

journal homepage: www.elsevier.com/locate/renene



Enhancement of biodiesel potential in cyanobacteria: using agro-industrial wastes for fuel production, properties and acetyl CoA carboxylase D (*accD*) gene expression of *Synechocystis* sp.NN

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A R T I C L E I N F O

Article history:
Received 31 December 2015
Received in revised form
8 February 2016
Accepted 11 February 2016
Available online 28 February 2016

Keywords:
Cyanobacteria
Biodiesel
FAME
GC–MS
Semi-quantitative RT-PCR

A B S T R A C T

In this study, two freshwater cyanobacteria, *Oscillatoria* sp. 50A and *Synechocystis* sp. NN have been evaluated for biodiesel production. Among the two cyanobacteria, *Synechocystis* sp. NN was isolated, identified by its 16S rRNA gene sequencing. Effects of sodium bicarbonate (SBC), tannery effluent (TE), coir pith (CP) and light stress (L1) on biomass and lipid production of *Synechocystis* sp. NN were studied. Result showed that maximum biomass productivity of 18.7 ± 0.9 mg/L/day (1.9 folds) was observed in TE supplemented BG-11 media than normal BG-11 media. Meantime, maximum lipid productivity of 2.6 ± 0.4 mg/L/day (1.4 folds) was observed in CP supplemented BG-11 media than normal media. Further, fatty acid composition analyses by GC–MS showed that C16, C18:1 in *Oscillatoria* sp. 50A and C16, C20:1, C22:1 in *Synechocystis* sp. NN were predominant and the fuel properties were also in accordance with the international standards. Besides gene expression of acetyl CoA carboxylase D of *Synechocystis* sp. NN, analyzed by RT-PCR revealed that transcripts of *accD* were up-regulated by 1.2–4.7 folds in different media conditions. The findings of this study showed that *Synechocystis* sp. NN can be utilized as a suitable feedstock that is amenable for cultivation using wastes as nutrient source.
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1. Introduction

Demand for alternative fuel is getting great deal of significance worldwide, owing to the excessive consumption of fossils (non-renewable resources) and global warming. Prolonged use of fossil fuels has resulted in increased green house gas emissions that have eventually led to climate change globally [1]. In the recent years, prices of petroleum have escalated due to their huge energy demand from developing countries, diminishing fossil reserves and geo-political instability in oil exporting countries [2,3]. Thus, exploring renewable feedstock for fuel production is a major task for researchers. Fuel from algae and cyanobacteria is one such important renewable biological source, which is being used in developed countries. In this context, cyanobacteria are perhaps the most attractive prokaryotic bacteria, as they utilize cheap nutrients like industrial wastes. Similarly, algae show higher growth and lipid production in wastewater, which translates to better environmental management as improper disposal of industrial waste such as tannery effluent (TE), which is rich in inorganic chloride, sulphide, ammonia, fats, surfactants, arsenic and chromium, poisons freshwater sources and affects terrestrial and aquatic life [4–6]. Currently, researches focusing on wastewater utilization for the development of higher algal biomass that can be used as a renewable feedstock for biofuel production, are gaining much importance.

Diesel and gasoline can be comfortably replaced with biodiesel and bio-ethanol respectively in vehicles, with fewer or no modification. They offer the dual benefit of maintaining engine performance while being produced from renewable feedstocks [7]. Biodiesel production involves a chemical reaction called trans-esterification in which triglycerides react with methanol or ethanol in the presence of a catalyst to yield fatty acid methyl esters (FAME) [8]. As large agricultural lands will be required for the cultivation of plant feedstocks, and since there is always the possibility of

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http://dx.doi.org/10.1016/j.renene.2016.02.038
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competition with food crops, production of biodiesel from plant sources has been getting lesser importance in recent decades [9]. Consequently, cyanobacteria and microalgae are being considered as a raw material for biodiesel production owing their copious benefits including CO_2 mitigation, land availability and high oil yield [10,11]. In contrast with microalgae, cyanobacterial lipids can be enhanced by over-expressing proteins in thylakoid membranes which in turn improve the biomass and photosynthetic rates in the log phase [12]. Biodiesel fuel properties greatly depend on the ester moiety, obtained from alcohol during the transesterification reaction and the fatty acid profile [13]. Thus, biodiesel produced from a green renewable feedstock would be the best solution for mitigating the mounting fuel demand and global warming crisis. This study was designed to characterize the fatty acid profile of two freshwater cyanobacteria and to evaluate the biodiesel fuel properties based on their fatty acid composition. Attempts were also made to enhance the biomass and lipid productivity in unicellular cyanobacteria *Synechocystis* sp. NN by supplementing the growth media with sodium bicarbonate (SBC), tannery effluent (TE), coir pith (CP), and light stress (L1). Furthermore, the *accD* gene expression was also analyzed under the conditions of light stress and waste supplementation using semi-quantitative RT-PCR.

2. Materials and methods

2.1. Cyanobacterial isolation and biomass cultivation

Cyanobacteria samples were collected from fresh water bodies in the vicinity of Madurai, Tamil Nadu, India and were enriched in BG-11 media [14]. Samples were serially diluted and streaked onto BG-11 agar plates and incubated for 15–20 days at 25 °C under constant illumination of alternate photoperiod (12 h light:12 h dark) at 1500 lux. Cultures were monitored microscopically and an axenic colony was isolated by repeated streaking. Axenic cultures were then transferred to 5 mL of BG-11 liquid medium and sub cultured to 100 mL BG-11 medium. An oleaginous unicellular cyanobacetrial isolate NN and a filamentous cyanobacterium *Oscillatoria* sp. 50A were subjected for further evaluation of biodiesel production in this study.

2.2. High resolution – scanning electron microscopy (HR–SEM)

In order to visualize the surface topology, isolate NN was imaged under HR-SEM (Carl Zeiss EVO 18, Germany). Cells were suspended in carbon tape and air dried. Filament was adjusted for optimum resolution in conducting mode under the magnification of 10,000X and the image was captured using Backscattered Electron Detector.

2.3. Genomic DNA extraction and molecular characterization

Genomic DNA was extracted from the log phase cultures of isolate NN using modified method [15]. 16S rRNA gene amplification was performed using genomic DNA (50 ng) as template and universal primers 27F and 1492R. Briefly, 50 µl reaction comprising 25 µl of ReadyMix™ Taq PCR Reaction Mix (Sigma–Aldrich, St. Louis, MO), 17 µl of molecular biology grade water (Hi–media, Mumbai, India), 2 µl of forward and reverse primers each (0.4 µM) and 4 µl of genomic DNA was used. 16S rRNA gene specific forward 5′ – AGAGTTTGATCCTGGCTCAG – 3′ and reverse primers 5′ – CGTTACCTTGTTACGACTT – 3′ were used. PCR was carried out in Nexus Gradient Mastercycler (Eppendorf, Germany) with the following PCR program: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. A negative control was also run along with PCR using sterile water as template. PCR products were separated by gel electrophoresis on 1.2% agarose gel for 30 min at 50 V in an electrophoretic apparatus. Amplicon with expected size was visualized under UV transilluminator by ethidium bromide staining and purified using GenElute kit (Sigma–Aldrich, St. Louis, USA). PCR amplicons were sequenced using Sanger's dideoxy sequencing method and subjected to BLAST (www.ncbi.nlm.nih.gov) to identify the homologous sequence by comparing the query coverage and e-value. The identified 16S rRNA gene sequence of isolate NN was submitted to GenBank (http://www.ncbi.nlm.nih.gov/).

2.4. Study of biomass and lipid enhancement in Synechocystis sp. NN

In order to enhance the biomass and lipid production in *Synechocystis* sp. NN, cells were grown in BG-11 medium was supplemented with sodium bicarbonate (10 mM, 20 mM and 30 mM), tannery effluent (TE; 1.3 mL/L, 2.7 mL/L and 4 mL/L), coir pith (0.6 g/L) and also subjected to light stress (2000 lux). TE was collected from Subramaniam & Co Leather Industry Pvt. Ltd, Dindigul, Tamil Nadu, India. An initial inoculum of 12 mg (dry cell weight) of *Synechocystis* sp. NN was equally added to the flasks containing respective supplements and maintained as foresaid culture conditions for 40 days. To identify the functional groups present in the TE, FT-IR analysis was performed and the key physico–chemical parameters of TE like pH, total dissolved solids and alkalinity were evaluated.

2.5. RNA extraction and cDNA synthesis

Frozen *Synechocystis* sp. NN cells were ground using mortar and pestle with liquid nitrogen and the cell lysate was used to extract the total RNA (RNeasy® Mini Kit, QIAGEN) by following the manufacturer's protocol. The extracted RNA was quantified using Biophotometer (Eppendorf, Germany) and its purity was verified by measuring absorbance at 260/280. cDNA synthesis was performed using 4.5 µg of DNAase treated total RNA using random hexamers of RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) by following the manufacturer's protocol.

2.6. Semi-quantitative reverse transcriptase – PCR (RT-PCR)

In order to study the gene expression of *Synechocystis* sp. NN under control BG-11 medium and lipid productivity enhanced media conditions, semi-quantitative RT-PCR was performed. 16S rRNA gene was used as an internal control. Primers used in this study were provided in Table 1. Briefly, a total of 25 µl reaction was performed comprising 12.5 µl AMPLIQON Master Mix, 8.5 µl of Nuclease Free Water, 1 µl of forward and reverse primers each (0.4 µM) and 2 µl of cDNA. PCR was carried out during exponential phase [initial denaturation of 94 °C for 4 min followed by 38 cycles of denaturation at 94 °C for 45 s (12 cycles for internal control gene) and annealing at 60 °C for 45 s]. PCR products were electrophoresed and visualized under gel documentation unit. Densitometry software ImageJ was used to analyze the difference in band intensities of *accD* and 16S rRNA genes.

Table 1
Primer sets used for semi-quantitative RT-PCR.

Gene	Primer	Sequence	Product size
accD	Forward primer	GGTGGAGCCAGAATGCAGG	153bp
accD	Reverse primer	CAACATGGCAAAGCTGGCAGT	
16S rRNA	Forward primer	TAAAGCGTCCGTAGGTGGCATTAC	143bp
16S rRNA	Reverse primer	TTCCCGATATCTACGCATTTCACC	

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