Isolation and selection of growth medium for freshwater microalgae Asterarcys quadricellulare for maximum biomass production
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ABSTRACT

The use of microalgae biomass as a suitable alternative feedstock for biofuel production has been promoted in the field of green biotechnology. In this present study, the microalgae were isolated from freshwater samples. The predominant strain was screened from the samples and grown in four different growth media, including modified Bold’s Basal Medium (BBM), modified CFTRI medium, BG11 medium and CHU medium, to find the suitable growth medium to enrich biomass production. In total three microalgae colonies were identified based on their colony morphology microscopically by using a light microscope. The predominant strain was confirmed as Asterarcys quadricellulare using 18S rRNA sequencing. The growth of microalgae was investigated based on parameters like dry weight, pigment composition such as chlorophyll a, chlorophyll b, carotenoid and lipid content in the microalgae. Among the four different media, modified BBM medium showed maximum dry weight (1.44 ± 0.015 g/L), chlorophyll a (23.07 ± 0.049 mg/L), chlorophyll b (16.76 ± 0.010 mg/L), carotenoid (8.92 ± 0.031 mg/L) and lipid content (375 ± 0.020 mg/L) on the 25th day of culture. The gas chromatography mass spectrometry (GC/MS) analysis showed the presence of major fatty acids stearic acid, palmitic acid and oleyl alcohol in the microalgae. Therefore the high lipid content and fatty acid profiles of Asterarcys quadricellulare are becoming a promising suitable strain for biofuel production with modified BBM medium.

Key words | biofuel, carotenoid, chlorophyll, growth medium, lipid, microalgae

INTRODUCTION

With a constant rising of the population worldwide, there is a huge demand for energy resources. Due to the depletion of fossil fuels and their rising prices, there is a need for alternative renewable energy sources. Biomass is a suitable alternative for a conventional energy source that could be sustainably developed in the future. Biomass energy is one of the most important components to reduce greenhouse effects and substitute for fossil fuels (Goldemberg 2000). First-generation biofuels can be produced from carbon-rich biomass food crop sources such as sugar beet, rapeseed, canola, and palm (Hanjalić et al. 2008). The second-generation biofuels are produced from nonfood feedstock lignocellulosic biomass that does not compete with food production. Now all over the globe, researchers are focused on third-generation biofuels derived from microalgae that overcome the major drawbacks associated with first- and second-generation biofuels (Li et al. 2008). Microalgae are the potential source for biofuel production due to their unique properties. The use of petroleum-based fuels is intimately linked with increasing emission of CO2 and global-warming effects (Malakootian et al. 2015). Microalgae are fast-growing photosynthetic organisms, rich in oil content, fixing atmospheric CO2 and thereby reducing global warming, nontoxic, containing no sulfur and highly biodegradable (Khan et al. 2018). Microalgae can grow in freshwater, seawater or brackish water and utilize the nutrients from all kinds of agricultural and municipal wastewater and industrial effluent and thereby play a crucial role in wastewater remediation (Beltrán-Rocha et al. 2017).

The major critical parameters which determine the economic feasibility of microalgae as biofuel feedstock, particularly in open pond cultivation, include lipid content, lipid productivity and biomass productivity. But accomplishing high lipid content with high biomass productivity is a
challenge (Coelho et al. 2019). It is very essential to screen and select the native high-lipid-producing microalgae species for biofuel application. Generally prior to mass production of microalgae in a given area, selection of indigenous microalgae from the locally isolated strains should be carried out (Lari et al. 2016). This is due to the fact that native isolates, which are well adapted to local conditions, exhibit better performance and robustness than those of the strains obtained from a bank collection. Although numerous microalgae strain collections have been established, still a variety of unknown new potential biofuel production strains are present in the environment (Jebali et al. 2019). So the search for novel useful strains from specific locations is important for definite applications.

There are several standard culture media reported to culture microalgae. Both physical and chemical parameters such as nutrient composition, temperature and light directly influence biomass production (George et al. 2014). The growth of any microalgae mainly depends on the quality of the medium used for their cultivation (Lam & Lee 2012). The concentration of nitrogen and phosphorus are the most important restrictive factors in microalgae growth media that can lead to the accumulation of lipids and carbohydrates (Malakootian et al. 2015). Therefore, the selections of suitable strain, growth medium and harvesting time are essential parameters to achieve high yields of desired microalgae products. Hence, in this present study, we isolated native microalgae species from stagnant water bodies. Out of several isolated strains, we selected a new single native novel fastest-growing strain and investigated the impact of its biochemical composition against different growth media.

MATERIALS AND METHODS

Sample collection

Microalgae samples were collected aseptically from different stagnant freshwater bodies during the rainy season (September to December) using a phytoplankton net (25 μm) in and around Chennai (12.9556° N, 80.1869° E), Tamil Nadu, India. All the water samples were collected and labeled in sterile plastic tubes and kept in refrigerated conditions.

Screening and enrichment of algal isolate

The stored freshwater samples were centrifuged at 5,000 rpm for 10 min, and the algal biomass was resuspended under aseptic conditions at 25 ± 1 °C in 50 mL of liquid Bold’s Basal Medium (BBM) for enrichment. It was maintained under 30 μE m⁻² s⁻¹ light intensity and photoperiods of 12 h/12 h light/dark cycle for 7–10 days (Stanier et al. 1971). To isolate individual microalgae colonies from the water samples, standard plating methods were employed to separate algal populations.

Isolation of microalgae

The enriched mixed microalgae culture samples were serially diluted (10⁻¹, 10⁻², and 10⁻³) with sterile Millipore water and streaking was done on 1.5% solid BBM agar plates under aseptic conditions. The visible and distinct colonies were picked with a sterile loop and subcultured in liquid BBM medium. The flasks were incubated on a rotary orbital shaker at 150 rpm under continuous illumination (30 μE m⁻² s⁻¹) using white fluorescent light for 15 days. The above method was repeated until the isolation of unialgal cultures was achieved, and the purity of the culture was regularly observed under an optical microscope (Andersen 2005). The identification of individual isolates was done based on the standard morphological features of microalgae (John et al. 2003).

Genotypic characterization of the screened isolate

The screened microalgae strain was primarily identified with a light microscope and the molecular confirmation was done by 18S rRNA sequence analysis. The total genomic DNA was initially extracted using a High Pure PCR template preparation kit (Roche). The presence of extracted DNA was analyzed by electrophoresis using 1% agarose. The 18S rRNA sequence of the microalgae was amplified using PCR (Applied Biosystems, USA) with the universal eukaryotic primers, SSU1: 5'-TGAGTGGTCTCTGCCAGTA-3' as a forward and SSU2: 5' TGATCCTTCGCCAG GTTCAC-3' as a reverse primer (White et al. 1990). The amplified PCR product was purified from the gel using a purification kit (MN Kit, USA). PCR products were electrophoresed with 1.0% agarose gel for confirmation.

Sequencing and taxonomic analysis

The sequence was performed by using an automated DNA sequencer (ABI 3730XL, Applied Biosystems). The generated 18S rRNA gene sequences were compared with the GenBank nucleotide database using the BLASTN for homologous analysis, according to similarities by the ClustalW program. BLASTN matches that aligned closely, between 90% and 99% with the screened microalgae strain, were
selected and added to sequence comparisons. Phylogenetic tree analysis was performed using the Molecular Evolutionary Genetics Analysis v6 (MEGA6) software (Tamura et al. 2013).

Axenization of microalgae culture

The screened microalgae isolate Asterarcys sp. was made axenic by the triple antibiotic treatment method (Droop 1967). The isolated strain was serially diluted in the presence of three antibiotics such as benzyl penicillin, chloramphenicol, and streptomycin with different concentrations and sterile BBM medium was used as the diluent. A sample of 2.0 mL of each treated culture was centrifuged at 5,000 rpm for 5 min. Then the algal samples were washed thoroughly with BBM and sub-cultured in the BBM without antibiotics. After 10 days of incubation, the purity of the isolated axenic cultures was checked by inoculating the algal culture in the medium containing: peptone – 10.0 g, yeast extracts – 1.0 g, BBM – 1,000 mL and pH 7.5. In addition, bacterial contamination was evaluated using a microscope.

Nutrient medium optimization

The purified axenized Asterarcys sp. culture was subjected to four different growth media for selection of a suitable medium providing the best growth under photoautotrophic conditions. The four different growth media were namely, (i) modified BBM medium (modified from Bischoff & Bold 1963): NaNO₃ – 2.94 mM, CaCl₂ – 0.23 mM, MgSO₄ – 0.30 mM, K₂HPO₄ – 0.45 mM, KH₂PO₄ – 1.29 mM, NaCl – 0.43 mM; (ii) modified CFTRI medium (Singh et al. 2002): NaHCO₃ – 11.9 mM, urea – 12.49 mM, K₂SO₄ – 13.37 mM, DAP – 0.46 mM, MgSO₄.7H₂O – 0.30 mM; (iii) BG11 medium (Rippka et al. 1979): NaNO₃ – 1.77 mM, K₂HPO₄ – 3H₂O – 0.25 mM, MgSO₄.7H₂O – 0.30 mM, CoCl₂.2H₂O – 0.15 mM, Na₂CO₃ – 0.02 mM, C₆H₆O₇ – 0.05 mM, C₆H₈FeNO₇ – 0.02 mM, EDTA (Na₂Mg salt) – 2.6 μM and (iv) CHU medium (Yamaguchi et al. 1987): KNO₃ – 3.67 mM, K₂HPO₄ – 0.46 mM, MgSO₄ – 0.81 mM, CaCl₂ – 2H₂O – 0.45 mM, FeC₆H₅O₂ – 0.08 mM, C₆H₆O₂ – 0.52 mM. The growth media were prepared based on their nutrient compositions, transferred into 100 mL conical flasks and sterilized at 121 °C for 15 min at 15 lb pressure. After inoculation, the microalgal cultures were exposed to continuous illumination (50 μE m⁻² s⁻¹) and incubated under 25 °C ± 1 °C, and light intensity 12/12 light/dark photoperiod for 50 days. The inoculated cultures were gently shaken three times a day to avoid clumping and accelerate the microalgal growth. At every 5-day interval, 5 mL of grown culture was drawn from all the four medium samples and various biochemical parameters such as chlorophyll a, chlorophyll b, carotenoid, lipid and dry weight were analyzed to determine the suitable growth medium for the screened algal isolate. Each experiment was carried out in triplicate and the resultant mean values were represented.

Dry weight determination

The growth of the microalgae was assessed through the optical density (OD) of the culture at 680 nm using UV–Vis spectrophotometer (Thermo Scientific) at every 5-day interval related to algal biomass (g/L). For microalgae biomass estimation, 10 mL of microalgae sample was filtered through pre-weighed Whatman Filter Paper No. 1 and dried in an oven (70 °C) until a constant weight was reached (Richardson & Grobbelaar 1986). The dry cell weight was calculated in terms of g/L. The specific growth rate (μ day⁻¹) of the microalgae was calculated by using Equation (1):

\[
\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1}
\]

where \(x_1\) and \(x_2\) are initial and final biomass concentration, while \(t_1\) and \(t_2\) are the time interval (Lee & Kim 2002).

Pigment analysis

Pigments chlorophyll a, chlorophyll b and carotenoid were extracted from microalgae grown in different culture media at five regular intervals from the 5th day to the 40th day. Cells were harvested using centrifugation at 5,000 rpm for 10 min. Subsequently, total chlorophyll and carotenoid contents were extracted in 100% methanol. The absorbance of the green supernatant was measured and estimated according to the Lichtenthaler (1987) method. The supernatant was collected and its OD recorded at 470 nm, 644.8 nm and 661.6 nm in the UV–Vis spectrophotometer. The amount of chlorophyll a (mg/L), chlorophyll b (mg/L), and carotenoid contents (mg/L) were calculated using the standard formulas (Lichtenthaler 1987):

\[
\text{Chl} \ a \ (\text{mg/L}) = 11.24 \times A_{661.6} - 2.404 \times A_{644.8}
\]

\[
\text{Chl} \ b \ (\text{mg/L}) = 20.13 \times A_{644.8} - 4.19 \times A_{661.6}
\]

Total carotenoids = \[
\frac{1000 \times A_{470} - 1.9 \times \text{Chl} \ a - 63.14 \times \text{Chl} \ b}{214}
\]
Extraction of lipids

Lipids were extracted from microalgae biomass using the Folch et al. (1956) method. The microalgae cells were harvested by centrifugation at 5,000 rpm for 15 min. The biomass was separated from the supernatant and washed with double distilled water and air dried. The pellet was disrupted with glass beads in a vortex mixer. The lipid was extracted with a chloroform and methanol mixture (2:1, v/v). The lower chloroform layer was washed with saline solution and the solvent was allowed to evaporate by using a rotary evaporator at room temperature. The dried total lipids were measured gravimetrically.

Transesterification

The extracted lipid from the microalgae was converted to biodiesel using NaOH as catalyst according to the protocol of Darnoko & Cheryan (2000). In a typical transesterification reaction, 10 g of microalgae lipids were added to 100 mL of preheated sodium methoxide solution in a round-bottom flask equipped with a reflux condenser. The reaction mixture was heated up to 60 °C with continuous stirring using a magnetic stirrer (500 rpm) and refluxed for 45 min. The mixture was separated into two distinct layers after settling down overnight. The lower layer containing glycerol was drawn off. The separated top layer comprising methyl esters was purified by washing with hot distilled water three times to remove the excess methanol, catalyst and traces of glycerol if any. The moisture present in the methyl esters was removed by adding anhydrous sodium sulphate. Finally it was dried in an oven at 110 °C to remove residual moisture content. The obtained biodiesel sample was analyzed using gas chromatography mass spectrometry (GC/MS) (6890N, Agilent Technologies, USA). The initial oven temperature was programmed at 60 °C for 2 min, and then increased to 300 °C at a ramp rate of 10 °C per min, held for 6 min, the solvent delay time maintained at 2.5 min and the injector and source temperature was 240 °C. The column dimension was 30.0 m×250 μm and helium was used as carrier gas.

Statistical data analysis

Each experiment was done in triplicate and the mean and ± standard deviation (SD) was calculated using GraphPad Prism 6 statistical software. One-way analysis of variance (ANOVA) with post hoc test (Tukey’s method) to a level of 5% (P < 0.05) was used to test for significant differences among the growth parameters, dry weight and biochemical composition of microalgae.

RESULTS AND DISCUSSION

Isolation and identification of microalgae

In the present study water samples with visible microalgae populations were collected from different freshwater bodies during the rainy season in Chennai, Tamil Nadu, India. The samples were serially diluted for the isolation of unialgal culture. The diluted samples were inoculated in sterile Bold’s Basal Medium for microalgae enrichment. The algal strain was streaked on BBM agar plates for obtaining individual colonies. After the enrichment, subculturing was carried out until the isolation of individual colonies.

Morphological identification

Microalgae cultures were initially separated based on morphological examination under the light microscope (Eclipse E200, Nikon, Japan). The microscopic observation confirmed the presence of three green microalgae species, namely, Asterarcys species, Chlorella species, and Scenedesmus species respectively. The visual identification of these species to the genus level was based on the standard morphological features of microalgae under the light microscope (John et al. 2003). After identification, the species were initially grown in BBM and subcultured for purification. Laboratory studies showed that among the three species, Asterarcys sp. showed the maximum biomass productivity (0.08 gL⁻¹d⁻¹) and higher lipid content (30.55% (w/w)). Asterarcys sp. was selected for further experimental studies (Figure 1).
Isolation of genomic DNA and PCR analysis

Morphological identification of microalgae based on microscopic examination is difficult due to the morphological heterogeneity of the algal cells, because within each chemical race the structural morphology of the microalgae may vary, depending on the culture conditions and age. Molecular marker-based identification of microalgae has been implemented in recent years to solve the taxonomic problem (Radha et al. 2013). The nuclear-encoded 18S rRNA gene of the green microalgae Asterarcys sp. was amplified by PCR using eukaryotic universal primers SSU1 and SSU2 (White et al. 1990). The amplified PCR product was purified (MN Kit, USA) and analyzed for its presence. The single band showing on 1% agarose gel confirmed the presence of an amplified product.

Sequencing and phylogenetic analysis

The purified PCR sample was then sequenced by an automated DNA sequencer (ABI, 3730XL, Applied Biosystems). The length of the amplified 18S rRNA gene sequence of the microalgae strain was ~1,692 bp. The 18S rRNA gene sequence was compared with other sequences in GenBank using the BLASTN algorithm. The BLAST results showed that the sequence had 99% similarity with Asterarcys quadricellulare (KNUA020) in the GenBank database which was previously sequenced by Hong et al. (2012). The sequence of the Asterarcys quadricellulare strain was further submitted to GenBank (KT280061). In order to strengthen the results after BLASTN analysis, the sequence was aligned using ClustalW and then phylogenetic tree analysis was conducted with the MEGA 6 program (Figure 2).

Effect of different growth media on biochemical composition

Microalgae growth is affected by various parameters such as media composition, photoperiod, light intensity, and temperature. Four different inorganic media with varying chemical compositions were studied in the present investigation to identify the best growth medium. The culture medium composition not only affects biomass productivity but also alters the biochemical composition of the microalgae and the yield of valuable products like lipids, carbohydrates, proteins, and pigments (Mandalam & Palsdon 1998). In general, the growth of microalgae depends on the availability of a nitrogen source and a phosphate source. The selected Asterarcys sp. was cultivated in four different culture media, the modified BBM, modified CFTRI, BG11 and CHU medium, to evaluate the effects of different media components on microalgae growth. The growth of Asterarcys sp. in a different medium was evaluated in terms of biomass (dry weight), chlorophyll a, chlorophyll b, carotenoid, lipid, and protein content. The modified BBM medium and CHU medium were found to greatly influence the growth of the selected microalgae strain followed by CFTRI medium and BG11 medium. One of the best parameters to monitor microalgae production is the estimation of growth, normally expressed in biomass, algal density, and pigment content over a certain period (Moura Junior et al. 2007). The maximum growth was supported by the modified BBM medium. Figure 3 shows the changes in dry cell weight (DCW) concentration of Asterarcys sp. cultivated in four different media. The highest DCW (1.44 ± 0.015 g/L) was found in the modified BBM medium on the 25th day of culture followed by CHU medium (0.826 ± 0.021 g/L). The increased cell dry weight was 51.7%, 42.7%, and 46.2% higher than BG11, CHU and CFTRI medium respectively. This can be explained in general by the BBM medium nutrient composition having great influence on the growth of freshwater microalgae (Iasimone et al. 2017). Al-Shatri et al. (2014) reported that culturing of Scenedesmus sp. in BBM medium reached maximum biomass concentrations when compared with CHU medium. Similar results were reported when culturing S. dimorphus in the BBM medium, which showed faster growth as compared with the MAN and BG11 medium. The amounts of nitrogen and carbon supply to the culture medium have a profound effect on the rate of biomass produced (Manikan et al. 2015). Other than carbon and nitrogen source, the amount of trace minerals present in the medium, especially iron and zinc, also affect biomass production to some extent (Nagano et al. 2015). The lowest DCW of 0.7 ± 0.009 g/L was recorded in the BG11 medium on the 25th day in the same culture conditions. The specific growth rate (µ day⁻¹) of microalgae increased until the biomass concentration reached the maximum value. The highest specific growth rate of microalgae of 0.1828 day⁻¹ was found in the BBM medium and was followed by CHU (0.1729 day⁻¹), BG11 (0.1626 day⁻¹) and CFTRI (0.1554 day⁻¹). Varshney et al. (2018) reported the specific growth rate of Asterarcys quadricellulare was 1.2 day⁻¹ under CO₂ and NO gas supplied growth conditions.

Changes in photosynthetic pigment concentration were analyzed to check the effects of different growth media on
The pigment composition of Asterarcys sp. The maximum concentration of chlorophyll $a$ (23.07 ± 0.049 mg/L), chlorophyll $b$ (16.76 ± 0.010 mg/L) and carotenoid content (8.92 ± 0.031 mg/L) were observed on the 25th day of culture in the modified BBM medium (Table 1). The concentration of chlorophyll $a$ in the modified BBM medium was 37.24%, 24.6% and 18.19% more than the values obtained from BG11, CHU and CFTRI medium respectively (Figure 4). On the other hand, the concentration of chlorophyll $b$ in the modified BBM medium was 37.24%, 24.6% and 18.19% more than the values obtained from BG11, CHU and CFTRI medium respectively (Figure 5). The lowest values of chlorophyll $a$ (14.44 ± 0.031 mg/L), chlorophyll $b$ (12.73 ± 0.025 mg/L) and carotenoid (6.58 ± 0.031 mg/L) content were recorded in the BG11 medium. The

![Phylogenetic tree demonstrating the closest relatives of isolate strain Asterarcys quadricellulare based on BLAST searches of 18S rRNA sequences.](image)

Figure 2

![Effect of different growth media on dry weight content of Asterarcys quadricellulare (g/L).](image)

Figure 3
carotenoid concentration in the modified BBM medium was 26.23%, 19.73% and 19.61% higher than in BG11, CHU and CFTRI medium respectively (Figure 6). MgSO₄ was the magnesium source that enhanced the production of pigments chlorophyll *a*, chlorophyll *b* and carotenoid content, thereby overall biomass was increased in the isolated microalgae (Mandalam & Palsson 1998). The highest lipid content of 375 ± 0.020 mg/L was found in the modified BBM medium followed by CFTRI (304.99 ± 0.015 mg/L) grown culture on the 25th day of culture (Figure 7). It was 31.2%,
21.3%, and 18.7% higher than that of the microalgae grown in BG11, CHU, and CFTRI medium respectively. The greater accumulation of lipids was obtained in the BBM medium due to the fact of nitrogen starvation conditions in the medium. Similar studies reported that when culturing Chlorella vulgaris under nitrogen starvation conditions, there was an increase in total lipid production (Li et al. 2008; Yeh & Chang 2011). Oliveira et al. (2011) reported 20% lipid yield was obtained in Asterarcys quadricellulare when cultivating in BBM medium. Similarly Hong et al. (2015) reported 15.5% lipid yield was obtained in Asterarcys quadricellulare KNUA020 when cultivating in BG11 medium. In this present study, the microalgae Asterarcys quadricellulare KT280061 showed the higher lipid yield of 25.2% in dry weight.

**GC/MS analysis**

The GC/MS analysis of A. quadricellulare shows that it has the combination of both saturated fatty acids and monounsaturated fatty acids (Figure 8). Stearic acid (C18:0) and palmitic acid (C16:0) were the major dominant saturated fatty acids ranging from 62.5% to 78.8% and followed by the monounsaturated fatty acid oleic acid (C18:1) and heptadecanoic acid (C17:0) ranging from 24.6% to 44.1% present in the microalgae. The other minor polyunsaturated fatty acids arachidonic acid (C20:4) and linoleic acid (C18:2) were also recorded in FAME content. The fatty acid composition of our Asterarcys sp. strain KT280061 is similar to the previously reported Asterarcys sp. (Odjadhare et al. 2018; Saber et al. 2018; Chaudhary & Khattar 2019). If the fatty acid methyl ester profile of biodiesel shows a mixed combination of saturated, monounsaturated, and polyunsaturated fatty acids, it is considered a good quality biodiesel according to ASTM standard D6751-07 (Bagul et al. 2017). The microalgae lipid profile contains a greater amount of oleic acid having a good balance of fuel in terms of oxidative stability, ignition quality and viscosity (Abou-Shanab et al. 2011). Therefore, the freshwater microalgae A. quadricellulare has become good for biofuel production to replace fossil-based fuels.

**CONCLUSIONS**

Due to the increase in the energy crisis and climate change, it is essential to develop alternative energy forms that are sustainable and environmentally friendly. Biofuel is a valuable alternative for fossil fuel and it has drawn more attention among researchers. The present work investigated the effect of four different media, modified BBM medium, modified CFTRI medium, BG11 medium and CHU medium, on the growth of screened microalgae. The modified BBM medium favours the maximum growth of Asterarcys quadricellulare with a biomass content of 1.44 ± 0.015 g/L and lipid content of 375 ± 0.020 mg/L. The results obtained in this work show that the freshwater microalgae Asterarcys quadricellulare is a promising strain for biofuel production. Furthermore, very few researchers have reported about this species, and still no experimental studies had been encountered concerning the medium optimization of Asterarcys sp. in the scientific literature. The high lipid content and fatty acid content of these freshwater microalgae offer new opportunities in biofuel-based applications. Further studies should be done to make use of these strains for large-scale mass cultivation under outdoor open pond conditions.
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