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DOMESTIC WASTEWATER TREATMENT AND LIPID ACCUMULATION FOR BIODIESEL PRODUCTION BY AN ISOLATED HETEROTROPHIC MICROALGAE FROM AN ARID CLIMATE ZONE

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ABSTRACT

The isolation of microalgae that can use carbon-rich pollutant from wastewater and accumulate lipids is of great interest in biodiesel production. The heterotrophic cultivation of microalgae could overcome light dependency, and hence increase the yield of microalgae lipid per unit area. After several microbial screening and acclimation procedures, strains of microalgae were proved to be tolerant of wastewater and to grow heterotrophically. One top-performing strain (AE2) was identified through morphological observation as Chlorella sp and was isolated from an open pond of domestic wastewater. This microalgae is able to grow in raw wastewater at 20°C with no illumination and eliminates 100% of its COD (424 mg/L) in 9 days. The biomass produced in wastewater as growth medium comprises 53% of fats of the dry mass and 68% of fats of the dry mass in BG 11 culture medium supplemented with 5 g of glucose/L. The analysis of fatty acid methyl esters FAME composition was 32.5 % of the total biomass. The extracted microalgae oil was converted to good quality biodiesel. This heterotrophic isolated microalgae is a promising strain in terms of wastewater COD removal and cytoplasmic accumulation of high quality and quantity lipids for prospective biodiesel production.

Contribution/Originality: This study is one of the very few studies which have investigated the isolation of microalgae from the southern climate that can grow efficiently in low carbon sources such as wastewater. The isolated microalgae produced biomass with 53% of fats that are favorable in term quantity and quality for biodiesel conversion.

1. INTRODUCTION

Different kinds of biomass containing forestry, agricultural, and aquatic sources have been explored as the feedstock for the production of different biofuels including biodiesel, bio-ethanol, bio-hydrogen, bio-oil, and bio-gas. Biodiesel, a renewable biofuel with similar combustion properties to fossil diesel, is normally produced by trans-esterification of oils with short-chain alcohols. Biodiesel can significantly decrease the exhaust emission of CO_2, SOx

and unburned hydrocarbons from motor vehicles. Biodiesel is environmentally beneficial, and therefore, a promising alternative to fossil diesel. The most abundant biodiesel is the first generation biofuel created from agricultural edible crop oils. It has an excessive influence on food security and has the potential to increase the cost of food crops such as soybean, thus making biodiesel production more expensive [1]. The second generation biofuels such as jatropha oil, waste cooking oil, and animal fats do not affect food security but are not sustainably resourceful. Microalgae biofuel is reflected as the third generation biofuels source. The lipid content of microalgae on a dry cellular basis generally varies between 20% and 40%; however, lipid contents as high as 65% have been reported for certain microalgae strains [2].

Important interest in algal-based biodiesel has been shown over the past few years due to the sharp rise in fossil fuel prices and increasing concerns about the global climate change [3]. However, growing algae for energy use is still debatable in terms of sustainability and economic viability of such algae-derived biofuels [2, 4]. Wastewater effluent as a nutrient medium would expand the sustainability of microalgae biofuels production [5]. In fact, up to date, there is a major price gap between microalgae-derived biofuels and fossil fuels despite the tremendous efforts to reduce the costs of algae production and processing. To overcome the cost obstacle and to make biofuels from microalgae economically feasible, at least three areas need to be explored: (1) finding of low- or zero-value carbon sources to support heterotrophic microalgae growth, (2) designing of appropriate bioreactors for industrial scale heterotrophic cultivation, and (3) identifying suitable pathways for converting algal biomass to biofuels [4].

Many algal organisms are capable of using either autotrophic or heterotrophic metabolism process for growth, and therefore able to photosynthesize as well as ingest organic materials [6].

Miao and Wu [7] also studied *C. protothecoides* and found that the lipid content in heterotrophic cells could be as high as 55%, which was 4 times higher than in autotrophic cells at 15 % under similar conditions.

Combining wastewater treatment with heterotrophic algae cultivation for biofuel production may offer an economically viable and environmentally friendly means for sustainable renewable algal based energy production since enormous amounts of water and nutrient (e.g. nitrogen and phosphorus) required for algae growth could be saved in such wastewater-based algal cultivation system [8, 9]. It is estimated that the biomass productivity of microalgae could be 50 times more than that of switchgrass, which is the fastest growing terrestrial plant [1, 10] and can be twenty times of oilseed crops on a per hectare basis and is thus a more viable alternative [11].

Microalgae have the natural capability to remove organic carbon, nitrogen and phosphorus from wastewater. Yet in terms of achieving the two purposes of wastewater treatment and lipid production simultaneously, no studies have so far demonstrated promising results. Isolating superior algal strains could be an option, but care needs to be taken to ensure their competitiveness against the native microbial species and robustness in surviving against temperature change in a natural setting [4]. One example is to select algal strains that are facultative heterotrophic, adaptable to the northern climate, able to consume organic carbon, nitrogen, and phosphorous in wastewater, and capable of high yield of biomass and lipid [4].

It is in this direction that our work aims at isolating vigorous microalgae from the southern climate that can grow efficiently in low carbon sources to support heterotrophic microalgal growth, such as wastewater, and accumulate adequate quality and quantity of lipids favorable for to biodiesel conversion.

2. MATERIALS AND METHODS

2.1. Isolation of Microalgae Strains

Microalgae were isolated from five different types of water bodies in Morocco (arid climate z), comprising creeks, ponds, and wastewaters. The samples were collected and stored in sterile transparent plastic. The protocol for isolating the unicellular algae strains was conducted based on the work of Zhou, et al. [11] using BG11 artificial medium [12].

2.2. Strains Screening

To identify strains with a larger growth on wastewater, two steps were piloted using solid media in Petri-dishes followed by liquid media in flasks on orbital shakers [11]. Features of morphology such as cell dimensions, cells shape, reproductive features, chloroplast shape and number were evaluated in 3-week-old cultures using a microscope (Olympus IX70, USA). They were compared with the published descriptions of Ettl and Gärtner [13].

2.3. Wastewater Samples

The wastewater was obtained from an open domestic wastewater stream. It has a high total suspended solid concentration and high turbidity, which reduces light transmission for algae cultivation. This wastewater was first centrifuged (5000 rpm, 10 min) to remove the suspended solids and then sterilized by autoclave to eliminate endogenous microorganisms. Wastewater analysis was performed following the Hach DR 5000 Spectrophotometer Manual [14] to determine the total nitrogen (TN), total phosphorus (TP), and chemical oxygen demand (COD). Chemical features of the wastewater are presented in Table 1.

Table-1. Characterization of the used wastewater samples from two different sources.

Measured parameters	(mg/L)
Chemical oxygen demand (COD)	424.24
TOC	275.60
Total nitrogen (TN),	3.278
Total phosphorus (TP)	0.04
Ammonium	24.4

2.4. Experimental Set-up and Determination of Algal Growth

The isolated microalgae were cultured in 150 mL domestic wastewater in 500-mL flasks. Each strain of microalga was inoculated by 2.5% (v/v) into BG11 medium. Erlenmeyer flasks were covered by aluminum foil to keep away from the light and cultured in 20 ℃ incubators. All tests were carried out in duplicate.

2.5. Oil Extraction

Cellular lipid content was determined following a procedure developed by Liang, et al. [15]. Briefly, 0.5 g dried cell pellet was transferred to a 7 ml chamber of a bead-beater (Bio- Spec Products, Bartlesville, OK, USA). This chamber was filled with 0.5 mm zirconium beads to approximately 5 ml. Methanol was then added to fill the rest of the chamber. After cells were disrupted by bead-beating for 2 min., the entire content was transferred to a 50-ml glass centrifuge tube. The chamber was washed twice using methanol (total 10 ml) to collect the algae residue. Chloroform was then added to the tube to make the chloroform/ methanol (2:1, v/v). The tube was shacked (vortex) for 5 min. and was allowed to stand for 24 hrs. After that, the tube was centrifuged at 4000g for 15 min. to remove the zirconium beads and algal solids. The supernatant was collected and the solvent was vaporized using Rotovap. Oil left in the flask without solvent was weighed to calculate oil content.

2.6. Analysis of Fatty Acid Methyl Esters

The fatty acid methyl esters (FAME) were analyzed based on the procedure published by Miao and Wu [7]; Zhou, et al. [11]. In short, algae cells were harvested through centrifugation and then dried with a freeze dryer (Savant Instruments Inc., USA) before analysis. Fatty acid content and composition analysis were performed by following two consecutive steps including preparation of FAME and GC–MS analysis. FAME were prepared by a one-step extraction trans-esterification modified method described by Indarti, et al. [16]. Dried algae samples (about 100 mg) were weighed into clean, 25 ml screw-top glass bottles, to which 10 ml mixture of methanol, concentrated sulfuric acid, and chloroform (4.25:0.75:5) were added. Trans esterification was carried out in a 90 °C water bath (Cole- Parmer, USA) for 90 min. Upon completion of the reaction, the chloroform layer containing

FAME was carefully collected and subjected to GC–MS analysis. The GC was equipped with a flame ionization detector and a DB-5-MS capillary column. The oven temperature was set at 80 °C and held for 5 min., then raised to 290 °C at a rate of 4 °C/min, and held at 290 °C for 5 min., while the injector and detector temperature were set at 250 and 230 °C, respectively. The carrier gas (helium) was controlled at 1.2 ml/min. Chromatographic data were recorded and integrated using Agilent data analysis software. The compounds were identified in the NIST Mass Spectral Database and quantified by comparing the peak area with that of the external standard (C18:2) (Sigma–Aldrich, MO).

2.7. Production and Quality Assessment of the Obtained Biodiesel

Biodiesel was made out of the extracted microalgae oil as a raw material through the chemical reactions of trans-esterification using methanol and potassium hydroxide as a chemical catalyst. The obtained biodiesel was analyzed with respect to the selected ASTM requirements such as, flash point, density, water content and calorific value.

3. RESULTS AND DISCUSSION

3.1. Isolation and Screening of the Heterotrophic Microalgae

According to the above-mentioned steps of purification and isolation procedure [11] ten strains of algae- like microorganisms capable of growing on BG-11 agar plate were isolated from the above-mentioned wastewater bodies. When grown on BG-11, most of the isolated colonies' color varies between dark green and dark blue. This suggests that they belong to unicellular chlorophyll containing microalgae. Then, the focus shifted to selecting favorable isolated microalgae for wastewater treatment and lipid accumulation. Screening heterotrophic microalgae was performed with respect to the four-step work developed by Zhou, et al. [11]. Through this screening, six isolates were able to grow heterotrophically, and just three (EA2, EA4, and EA5) of them were able to grow on wastewater (results are not shown). It has been described that unicellular microalgae have been shown to be particularly tolerant to many wastewater conditions and very efficient in removing nutrients from wastewater [16]. These three strains were actually isolated from wastewater environment and concur with other works [11] stating that algae isolated from wastewater treatment plant sites or real water bodies can usually adapt to culture conditions similar to where they are found and grow better. The most performing microalgae in terms of maximal growth rate and biomass productivity (Figure 1) named AE2 was closely similar to *Chlorella* sp. strain based on morphologically features. This strain was the only one used as a biological tool in the remaining work. The growth data in terms of optic density (OD) for the species that could survive wastewater is summarized in Figure 1.

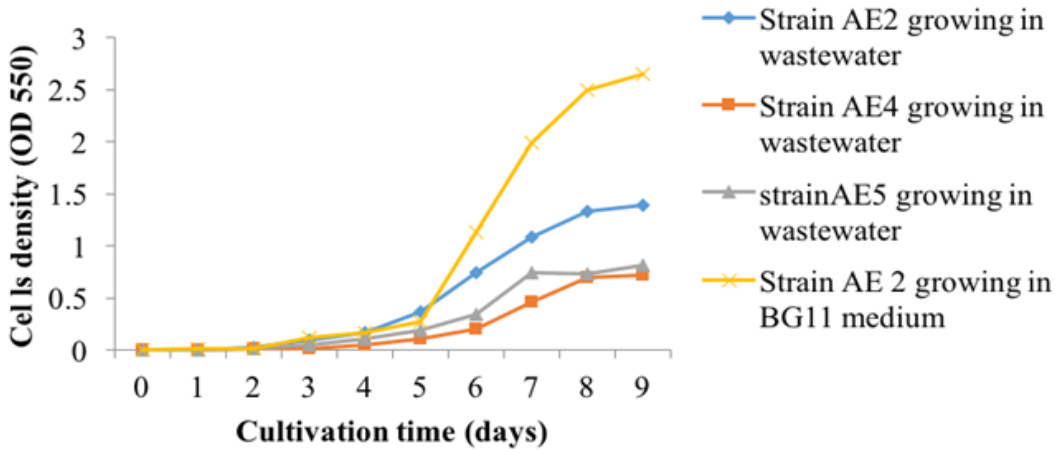

Fig-1. The changes of algal density curves during the cultivation of the three heterotrophic microalgae strains (AE2, AE4, and AE5) in domestic wastewater and AE2 in BG11 medium under dark condition and at 20°C. Measurements were done in triplicate, and the deviations from the average were less than 10%

3.2. Biomass Productivity and COD Removal

The composition of the used wastewater presented in Table 1: organic carbon, nitrogen, and phosphorus could lead to an imbalance in water nutrients, further triggering enormous growth of blue-green algae which is harmful to the environment [5]. It might support the growth of microalgae and replace artificial and expensive media under light/dark condition. Therefore, it was hypothesized that it could be used as substrate to replace artificial media and to support chemo-heterotrophic and photo-heterotrophic growth for candidate strains under light/dark condition [12] . AE2 was grown in the autoclaved and filtered wastewater for more than 9 days to evaluate their growth rate and biomass concentration. The growth reached the stationary phase during the first eight days with a considerable long lag phase with an OD ranging from 0.73 to 1.38 in wastewater and 2.6 OD in BG11 medium with 5 g glucose/L. Artificial medium BG-11 favors the indigenous isolates growth. Wang, et al. [17] measured the growth of *chlorella sp.* for ten days in four types of wastewaters and observed the stationary phase after 3rd day of cultivation time and lasted for next six days. This long lag phase could be explained by the initial higher COD. Growth became stationary on 7th and 8th day in synthetic medium and wastewater respectively and it is similar to the work of Farook, et al. [18].

This indicates that the dissolved matters in wastewater were utilized by microalgae and converted into the biomass with the increment of algal cells. These results are higher than those obtained by Zhang, et al. [19]. However, strains AE4 and AE5 presented a slight faster growth rate during the first days in wastewater as a medium (Figure 1). Their growth in BG-11 as medium was slow and it is not shown.

The initial COD (424.24 mg/L) of the wastewater was totally eliminated (100%) in nine days for AE2. However, AE4 and AE5 reduced the COD by 70% and 81% respectively (Figure 2). This, confirmed that the total organic compounds were completely consumed by the AE2 but partially by AE4 and AE5. These results showed a higher COD removal efficiency (up to 100%) compared with the work of Zhang, et al. [19] where the initial COD is 42.0 mg/L and the removal efficiency is about 40%. The top ranked strain AE2 with higher reduction of organic compounds in nine days seems to be a very prominent microalgae strain in removing organic compounds.

COD and BOD reductions of 91% and 51% respectively, were achieved over a period of 10 days in Nualgi-enriched samples [20].

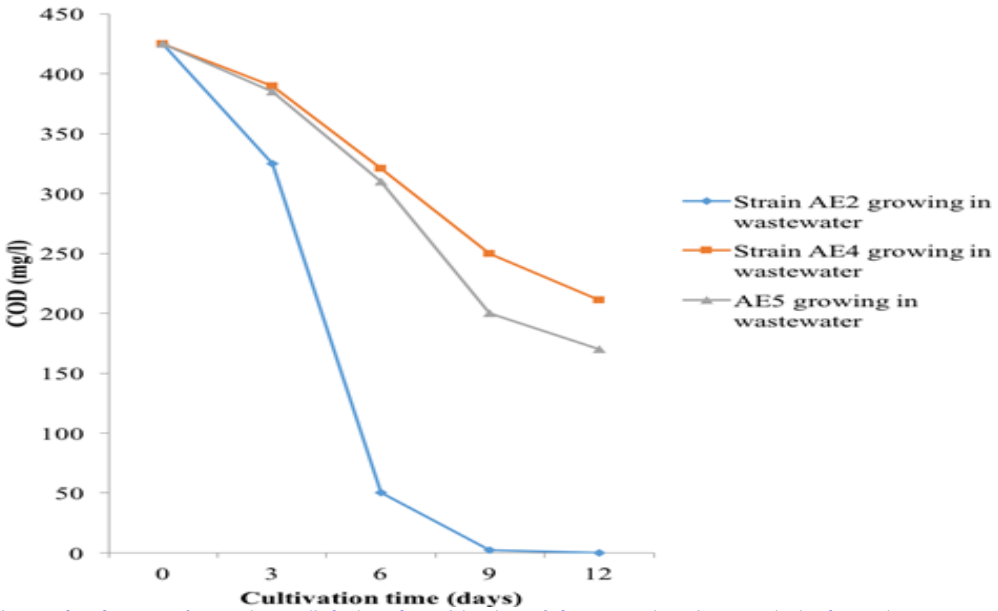

Fig-2. The changes of COD in mg/l during the cultivation of the AE2 microalgae strain in domestic wastewater under dark condition and at 20°C. Measurements were done in triplicate, and the deviations from the average were less than 10%

3.3. Lipids Accumulation and COD Removal

After 10 days of cultivation, the lipid content per biomass (%) of the heterotrophic microalgae strains AE2 in domestic wastewater and in BG11 medium under dark condition were determined (Figure 3). The microalgae used in this study indicated high lipid contents of 53% in wastewater and 68% in BG11 after nine days of cell culture at 20°C. These results are twice higher than those obtained in the work of Zhou, et al. [11] with a total lipid contents ranged from 17.41% to 33.53% of TVSS based weight. However, the starting COD concentration of the domestic wastewater in Zhang's study [19] was 40 mg/L, which is a much-diluted wastewater and with a COD removal of about 50%. Similar work carried out by Mahapatra, et al. [21] with Euglena sp. isolated from Facultative pond (STP) showed only a 24.6% of lipids. Yet EA2 was able to grow on domestic wastewater of 424.24 mg/L and eliminate it totally in nine days with a lipid content of 53% in wastewater.

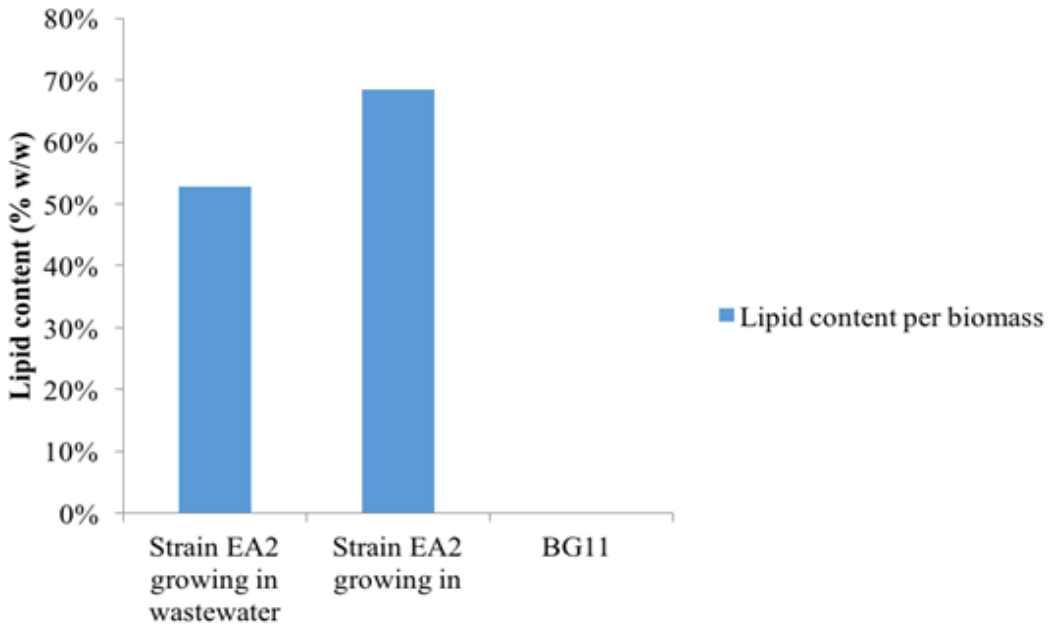

Fig-3. Lipid content per biomass of strain EA2 growing in wastewater and in BG11 medium. Measurements were done in triplicate, and the deviations from the average were less than 10%

3.4. Comparison of Lipid Content and Fatty Acid Profile of Candidate Microalgae

This procedure of fatty acid profile is used in conjunction with other procedures to determine the amounts of biofuel-relevant fatty acids present in algal biomass. As shown in Table 2. GC–MS analysis indicated that the main fatty acid components of the isolated microalgae AE2 were composed of C16–C18 fatty acids, accounting for more than 96.42% of total fatty acids. These C16–C18 fatty acids are suitable for biodiesel production [7, 22]. The top four fatty acids are: C18:1n9 (Oleic acid), C16:0 (palmitic acid), C18:2n6 (Linoleic acid) and C18:0 (Stearic acid). Among all fatty acids in the algal cells, these four accounted for 46.64%, 28.55%, 13.54% and 7.69%, respectively. Although higher un-saturation is not ideal for biodiesel production, the total saturated fatty acids was 36.7%, as for monounsaturated and polyunsaturated fatty acids, the numbers were 48.1 and 15.2%, respectively. Based on an internal standard of C17 that was used during the trans-esterification procedure, the total FAME was 32.5% of the total biomass.

It was found that the extracted oil from *chlorella vulgaris* [18] contained both saturated and unsaturated fatty acids but the percentage of unsaturated fatty acids (77.85%) was much higher than its saturated fatty acids percentage (21.15%).

Table-2. FAME profile of the algal cells

Name	Amount (% in oil)	Standard deviation (%)
C14:0 - Myristic acid	0.46	0.02
C16:0 - Palmitic Acid	28.55	0.68
C16:1n7 - Palmitoleic acid	0.84	0.08
C16:2n4 - Hexadecadienoic acid	0.05	0.01
C18:0 - Stearic acid	7.69	0.02
C18:1n9 - Oleic acid	46.64	0.39
C18:1n7 - Cis-vaccenic acid	0.38	0.05
C18:2n6 - Linoleic acid	13.54	0.09
C18:3n3 - Linolenic acid	0.03	0.03
C18:3n4 - Octadecatrienic acid	0.80	0.00
C18:4n3 - Octadecatetraenoic acid	0.02	0.01
C20:1n9 - Eicosenoic acid	0.19	0.03
C20:4n3 - Eicosatetraenoic acid	0.04	0.03
C20:4n6 - Arachidonic acid	0.09	0.05
C20:5n3 - Eicosapentaenoic acid	0.02	0.00
C22:5n3 - Docosapentaenoic acid	0.46	0.12
C22:6n3 - Docosahexaenoic acid	0.20	0.21

3.5. Conversion of Extracted Oil to Biodiesel

Biodiesel was washed by water to remove impurities (excess of catalyst and alcohol) and then its flash point, density, water contents and calorific value of biodiesel was determined. Results showed that all the parameters were within the limits of ASTM standards (Table 3)

Table-3. Comparison of the biodiesel produced from the isolate AE2 with the international standards ASTM

Properties Standards	Units	Produced Biodiesel	ASTM
Calorific value	MJ/kg	35	-
Flash point	°C	160	>130
Water content	% Vol.	0.04	0.05%
Density at 15 °C	g/cm^3	0.880	**0.85-0.90**

4. CONCLUSION

This work led to the isolation of a potential heterotrophic microalgae strain. It shows a potential to solve a number of important bottlenecks and challenges that algal biotechnology is facing, especially:

- Improvement of total biomass, lipid production and optimization of fatty acids composition. This indigenous strain has high biomass and lipid productivities and quality without the need for genetic modification.
- Use of carbon waste biomass as alternative, sustainable and renewable nutrient supply.

The isolate shows a high wastewater nutrient removal efficiency and confirms that wastewater effluent can be used as a microalgae culture platform for highly efficient biomass production.

The isolated strain could potentially be used in cultivation on other wastewater sources, such as industrial and agricultural wastewaters for perspective studies.

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