Sains Malaysiana 51(8)(2022): 2547-2557
http://doi.org/10.17576/jsm-2022-5108-15

Fatty Acid Profiling and Physiochemical Characterization of *Chlorella sorokiniana* Potentially Used for Biofuel Production

(Pemprofilan Asid Lemak dan Pencirian Fisiokimia *Chlorella sorokiniana* Berpotensi Digunakan untuk Pengeluaran Bahan Api Biologi)

MUHAMMAD AMMAR[1], MARIA OMER[2], SITWAT AMAN[2,3], ABDUL HAMEED[4], SAFDAR ABBAS[1], SHABNAM SHAHEEN[5], AIMAN ABBAS[6] & SAMINA N SHAKEEL[1,3,*]

[1]*Department of Biochemistry Faculty of Biological Sciences Quaid-i-Azam University Islamabad Pakistan*
[2]*Institute of Molecular Biology and Biotechnology University of Lahore Pakistan*
[3]*Department of Biological Sciences, Dartmouth College Hanover NH USA*
[4]*Genomic Lab, Main Saidpur Road, Rawalpindi, Pakistan*
[5]*Department of Higher Education, Government Girls Degree College Serai Naurang (Lakki Marwat), Peshawar, Pakistan*
[6]*University of Veterinary and Animal Sciences, Lahore, Pakistan*

Received: 20 November 2021/Accepted: 28 February 2022

ABSTRACT

Rising oil prices and climate change have resulted in more emphasis on research into renewable biofuels. In this study, different water samples were collected from local vicinities for the isolation of local isolates of microalgae to check their potential towards the production of biofuel by the addition of different chemical substrates. Five different concentrations of ascorbic acid and iron (III) chloride (0, 1, 2.5, 5 & 10 µM) are used as substrates. Microscopic analysis evaluated that samples belong to genus *Chlorella* and further molecular identification showed that the samples are *C. sorokiniana*. Among all the concentrations of ascorbic acid 2.5 µM is most effective against the *C. sorokiniana* strain 1 (Safari Wildlife Park, Lahore) and *C. sorokiniana* strain 2 (Bahria Town, Lahore) while *C. sorokiniana* Strain 3 (SukhChane Society, Lahore) responded at 2.5 & 5 µM in term of biomass production. $FeCl_3$ (2.5 µM) is effective against *C. sorokiniana* strain 1 while the growth of *C. sorokiniana* strain 2 and *C. sorokiniana* Strain 3 is inhibited. Lipid content analysis showed that only the *C. sorokiniana* strain 1 shows effective results at 1 & 2.5 µM of ascorbic acid and $FeCl_3$, respectively. Those concentrations which give the significant results of lipid production were preceded for fatty acid profiling. Results indicate that the *C. sorokiniana* strain 1 can be considered as a source of alpha-linolenic acid; the basic constituent of biofuel production. In this study, it is concluded that *C. sorokiniana* strain 1 is useful for the production of environment friendly biofuel.

Keywords: Ascorbic acid; fatty acids; free fatty acids; gas chromatography mass spectrophotometry

ABSTRAK

Kenaikan harga minyak dan perubahan iklim telah menyebabkan penyelidikan berkaitan biobahan api yang boleh diperbaharui diberi lebih penekanan. Dalam kajian ini, sampel air yang berbeza telah dikumpul daripada kawasan sekitar untuk pengasingan mikroalga dan memeriksa potensinya terhadap pengeluaran bahan api biologi dengan penambahan substrat kimia yang berbeza. Lima kepekatan berbeza asid askorbik dan besi (III) klorida (0, 1, 2.5, 5 & 10 µM) digunakan sebagai substrat. Analisis mikroskopik menilai bahawa sampel tergolong dalam genus *Chlorella* dan pengecaman molekul selanjutnya mendedahkan bahawa sampel tersebut adalah *C. sorokiniana*. Antara semua kepekatan asid askorbik 2.5 µM adalah paling berkesan terhadap *C. sorokiniana* strain 1 (Safari Wildlife Park, Lahore) dan *C. sorokiniana* strain 2 (Bahria Town, Lahore) manakala *C. sorokiniana* Strain 3 (SukhChane Society, Lahore) bertindak balas pada 2.5 & 5 µM daripada pengeluaran biojisim. $FeCl_3$ (2.5 µM) berkesan terhadap *C. sorokiniana* strain 1 manakala pertumbuhan *C. sorokiniana* strain 2 dan *C. sorokiniana* Strain 3 dihalang. Analisis kandungan lipid mendedahkan bahawa hanya *C. sorokiniana* strain 1 menunjukkan hasil yang berkesan masing-masing pada 1 & 2.5 µM asid askorbik dan $FeCl_3$. Kepekatan yang memberikan hasil ketara penghasilan lipid telah didahulukan untuk pemprofilan asid lemak. Keputusan menunjukkan bahawa *C. sorokiniana* strain 1 boleh dianggap sebagai sumber asid alfa-linolenik; juzuk asas pengeluaran bahan api biologi. Dalam kajian ini, disimpulkan bahawa *C. sorokiniana* strain 1 berguna untuk pengeluaran bahan api biologi mesra alam.

Kata kunci: Asid askorbik; asid lemak; asid lemak bebas; kromatografi gas spektrofotometri jisim

Introduction

Energy demand is dramatically increasing in the world and transportation sector consumes more than half of all fossil energy available. This sector is responsible for the majority of greenhouse gas (GHG) emissions into the atmosphere, which contribute to global warming. The cost and environmental changes concerns have focused more on the search for renewable biofuels (Spolaore et al. 2006).

A well-known area for such implementations is biodiesel derived from microalgae, suggested as a substitute for the fuels derived from fossils which are non-renewable (Saravanan et al. 2020) and microalgae are becoming increasingly relevant as one of the prospective sources for manufacturing biofuels. Biofuel is a form of fuel acquired from mammalian or plant oils and in addition methyl esters of long-chain unsaturated fats which are produced by the procedure known as trans-esterification of lipids. The lipids and other beneficial chemicals produced by microalgae may be employed in a variety of other applications, such as the creation of high-value compounds for medicinal treatments and health care items (Hasan & Rina 2012; Miao & Wu 2006; Munir et al. 2015).

Distinctive techniques are used for the growth of microalgae, which include use of different cultivation media, use of photo-bioreactors, resulting in a high yield. Those microalgae which are grown under stress conditions are involved in the production of some vitamins, lipids and pigments which are considered as secondary metabolites. These secondary metabolites are considered as high value byproducts (Singh et al. 2016), these are beneficial for different industries either it is a food, cosmetic or pharmaceutical field.

Fatty acids are one of the main components of biomass acquired by microalgae. These fatty acids are mainly present in the form of glycerolipids and those fatty acids which are present in the form of TAG are of economic importance due to their property of use in the production of fuel, food products and pharmaceutical grade nutrients such as omega-3 fatty acids (Breuer et al. 2012; Draaisma et al. 2013; Milledge 2010; Seo et al. 2015; Sydney et al. 2011). Microalgae are mainly involved in the production of those fatty acids which contain 16-18 carbon atoms in chain length. Both saturated and unsaturated fatty acids are produced by microalgae. Different analytical techniques are required for quantifying constituents of FA and their composition (Breuer et al. 2012; Draaisma et al. 2013).

Chlorella is among the most important microalgae used on commercial scale. High protein constituents, as well as a bulk amount of carbohydrates along with lipids are accumulated under different abiotic stresses. This is of great interest in biofuel production and other byproducts such as starch. *Chlorella* is also important for the production of other byproducts of economic importance such as starch, in this way an important alternative source for the production of bioethanol (Brányiková et al. 2011). The limited production of these chemicals in the natural environment, as well as the difficulties in isolating these products using cost-effective techniques, are the reasons behind this (Skjånes et al. 2013). Biomass extracted from *Chlorella* is rich in carbohydrates, minerals and other bioactive components; providing a good source for raw material used for feed and food. Still, progress has been still in process till full satisfaction in the field of genetically engineering chlorella strains (Hughes et al. 2012).

Microalgae which are grown under the optimal growth conditions are involved in the production of large amount of biomass, but the lipid constituents are low. Now the main challenge is to produce lipids meanwhile the high growth rate is also maintained for biofuels. The products after lipid extraction must be utilized as co-products (Parsaeimehr et al. 2015). High biomass production increases the yield per volume although a high lipid constituent decreases the expense of extraction per unit of the product. Different methods are suggested and practiced in economical viable microalgae to increase the production of bio-products, such as chemicals are used as a trigger for metabolism (Griffiths & Harrison 2009). Modern review study conducted by Franz et al. (2013) proposed phenotypic involvement of 42 substrates having role in lipid production in microalgae, in addition 12 chemicals are identified which are responsible for enhancing the intracellular lipid contents by >100% (Franz et al. 2013).

Local factors need to be considered in algal strain selection. For economic production of microalgal biodiesel, maximum lipid productivity should be achieved. It is possible by selecting suitable strains and culture conditions for enhancing growth rate as well as lipid contents of the cell. The current study was conducted to screen the isolated native microalgae strains in order to categorize with abiotic stresses on the biomass and lipid accumulation. Different concentrations (0, 1, 2.5, 5, & 10 μM) of two different chemical substrates were used to check out their phenotypic and molecular involvement in the production of biofuel derived from microalgae

ascorbic acid and iron III chloride.

Materials and Methods

Sample collection

After the collection of microalgae from different areas in vials containing Bold Basal Medium (BBM) were concentrated to 20 mL afterwards and were preserved in 10% buffered formalin.

Isolation of microalgae

For the isolation of single cell, the micro-algal samples initially grown in BBM. The cells were picked up and were placed on the glass slide containing BBM and observed under microscope. The samples were transferred to the next slide and this procedure was repeated for the isolation of single cell (Andersen & Kawachi 2005; Duong et al. 2012).

The single cell isolated was allowed to grow in Tris acetate medium under controlled conditions (Kropat et al. 2011). The sample was also grown on agar plates separately for preservation of sample.

Agar media was prepared by dissolving 2% agar (w/v) in Tris acetate phosphate medium, and sterilized by autoclaving at 121 °C for 15 min. After cooling down to room temperature, the media was poured in Petri plates and kept in incubator for 24 h to check the growth of contamination before streaking. 12 mL of each sample was taken in falcons and centrifuged at 3000 rpm for 15 min. The pallets were suspended in sterile water by vortex and this process was repeated to drive out other micro-organisms. Microalgae were shifted to agar plates by streaking and kept under controlled conditions for 10 to 14 days and the process were repeated until the isolation of single colony (Gigova et al. 2012). Microalgae were grown in Tris-acetate-phosphate (TAP) medium in 250 mL flasks under 16 h light/8 h dark cycle at 22-23 °C.

Molecular identification

DNA extraction: For the isolation of DNA CTAB (hexadecyltrimethylammonium bromide) method was used. The microalgal pellet was obtained by 300 rpm for 5 min and 500 µL of CTAB (hexadecyltrimethylammonium bromide) was added. After the incubation at 65 °C for 1 h, one volume of chloroform-isoamyl alcohol (24:1) was added to the sample and mixed by inversion. The sample was centrifuged for 30 min at 13,200 rpm and aqueous phase was collected, two volumes of absolute ethanol and 0.1 volume of sodium acetate 3M pH 5.2 was added and mixed. Incubation at -20 °C for 20 min followed by centrifugation at 13,200 rpm for 30 min and the supernatant were discarded. The palette was washed with 70% ethanol. Palette was air dried and re-suspended in 10-50 µL and sample were run in 1% agarose gel against known marker. The primers were designed from highly conserved 18s rRNA region. The forward primer (5`-TCCTGAGTAACGAACGAGACC-3`) and reverse primer (5`-CACGATGAAATTTCCCAAGAT-3`) contained the sequences to bind to genes. Polymerase chain reaction (PCR) was performed for the amplification of DNA fragment. Master Mix (buffer, dNTPs, MgCl2, forward primer, reverse primer, Taq. polymerase and water) was prepared and sample DNA was added, band quality and size of DNA fragment was checked by Agarose gel electrophoresis.

Assessment of effects of chemical substrates on growth of microalgae

Ascorbic acid and iron III Chloride were added to TAP (Tris-acetate-phosphate medium) in different concentrations (0, 1, 2.5, 5, & 10 µM) to check their effects on biomass and lipid accumulation on microalgae. Algae at stable phase (3 days) were collected in 50 mL tubes and centrifuged at 3000 rpm for 5-7 min. Supernatant was discarded and 5 mL of HCl was added following the incubation at 70 °C for 20 min. Samples were cooled down to room temperature, then added 5 mL ethanol and 10 mL of anhydrous diethyl ether. Afterwards vortex for 1 min then centrifuged at 3000 rpm for 5-7 min. Upper oil layer was collected and 10 mL of methanol was added and proceeded for evaporation through rotary evaporator (Ngangkham et al. 2013).

Physiochemical characterization

The upper oil layers collected from each sample in total lipid extraction were preceded for pH by using a pH meter. For the determination of Acid value, the sample was titrated against sodium hydroxide. After taking 20 mL of oil sample ethanol-ether solution was added and refluxed until the substance was completely dissolved. The solution was titrated until persistent pink color was observed for 30 s. The volume of sodium hydroxide titrant was measured, and the acid value was calculated by using the following formula (Milledge 2010):

$$\text{Acid value} = V_{NaOH} \times 5.61 \div W$$

$$\text{Density of sample} = M2\text{-}M1 / M3\text{-}M1$$

Trans-esterification

The obtained lipids from the sample 3 mL of methanol

containing 5% (v/v) sulfuric acid was added in that falcon. Incubation at 70 °C for 3 h followed by cooling down to room temperature and 3 mL of sterile distilled water and 3 mL of n-hexane was added. The samples were centrifuged at 12000 rpm for 5 min and upper layer of n-hexane was collected.

ANALYSIS OF GROWTH RATE, BIOMASS AND TOTAL LIPID CONTENT

The growth of microalgae was monitored by spectrophotometer device at optical density of 680 nm. The lipids extracted 3 days past the stationary phase and dry weight was obtained at the same time of lipid extraction. The collected samples were preceded for fatty acid profiling by gas chromatography (GC). For statistical analysis, a randomized complete block design (RCBD) was used, group means was compared by one-way ANOVA using graph pad.

RESULTS

MOLECULAR IDENTIFICATION OF *CHLORELLA* STRAINS

The genomic DNA was used as template for Polymerase chain reaction (PCR) and the conserved region of 18S rRNA was amplified as internal control. The results showed no difference of expression and a stable expression of that gene. The sequenced results of BLAST showed that sequence similarity of our three strains with already reported sequences of *Chlorella sorokiniana* in NCBI (Table 1).

TABLE 1. BLAST results of newly sequenced 18S rRNA gene of three strains showing similarity with other 18S rRNA genes of different microalgae

Accession	Description	Max score	Total score	Query coverage	E value	Max identity
KU948990.1	Chlorolasorokiniana isolate 19-4 18S ribosomal RNA	742	742	97%	0.0	98%
GQ487221.1	Heynigiadictyosphaerioides strain CCAP 222/2D 18S ribosomal RNA	742	742	97%	0.0	98%
KF887344.1	Micractiniumr eisseri clone EdL_Cl1_ MAF 18S ribosomal RNA gene	738	738	97%	0.0	98%
GQ507371.1	Dictyosphaerium sp. CB 2008/108 18S ribosomal RNA gene, partial sequence;	738	738	97%	0.0	98%
JX446472.1	Chlorellaceae sp. WJT36VFNP23 18S ribosomal RNA gene, partial sequence	758	758	96%	0.0	99%
HQ111430.1	Chlorella pulchelloides strain EN 2003/25 18S ribosomal RNA gene,	758	758	96%	0.0	99%
KF661334.1	Chlorella thermophila strain HTA1-65 18S ribosomal RNA gene, partial sequence	643	643	78%	2e-180	93%
JN090876.1	Uncultured eukaryote clone KRL01E16 18S ribosomal RNA gene, partial sequence	636	636	73%	3e-179	95%
HQ111430.1	Chlorella pulchelloides strain EN 2003/25 18S ribosomal RNA gene, partial sequence	787	787	97%	0.0	99%
KU926336.1	Ettliaoleoabundans strain rsemsu Neo-ol 18S ribosomal RNA gene, partial sequence	783	783	97%	0.0	99%

ASSESSMENT OF BIOMASS PRODUCTION BY THE ADDITION OF CHEMICAL SUBSTRATES

Each isolated strain gave different results for biomass at different concentrations of ascorbic acid and $FeCl_3$ (Figure 1) The *C. sorokiniana* strain 1 gave the maximum growth at a concentration of 2.5 µM of ascorbic acid (74.7±4.16 mg/mL as compared to that of control which is 69.0±2.00 mg/mL) and $FeCl_3$ (32.7±3.79 mg/mL as compared to control which is 23.7±2.31 mg/mL for the) (Figure 1 Strain 1) *C. sorokiniana* strain 2 gave maximum growth at the same concentration of ascorbic acid (62.5±2.18 mg/mL as compared to control which is 25.8±0.764 mg/mL), while the growth is inhibited by the addition of $FeCl_3$ (less than the control sample which is 43.0±3.00 mg/mL) (Figure 1 strain 2). In case of 3rd strain of *C. sorokiniana* the maximum growth was observed at a concentration of 2.5 & 5 µM ascorbic acid (61.0±2.65 mg/mL in comparison with control 39.0±2.65 mg/mL) while the growth was inhibited by the addition of $FeCl_3$ (less than the control 84.3±3.21 mg/mL) (Figure 1 Stain 3).

Effect of different concentrations of Ascorbic Acid and Effect of different concentrations of Iron III Chloride ($FeCl_3$) on biomass productivity of *C. sorokiniana* Strain 1(Safari Wildlife Park, Lahore), *C. sorokiniana* Strain 2 (Bahria Town, Lahore) and *C. sorokiniana* Strain 3 (Sukh Chane Society, Lahore). The data expressed as mean ± standard deviation of the three test (n=3). Significant differences were observed at different concentrations of chemical substrates ($p<0.05$).

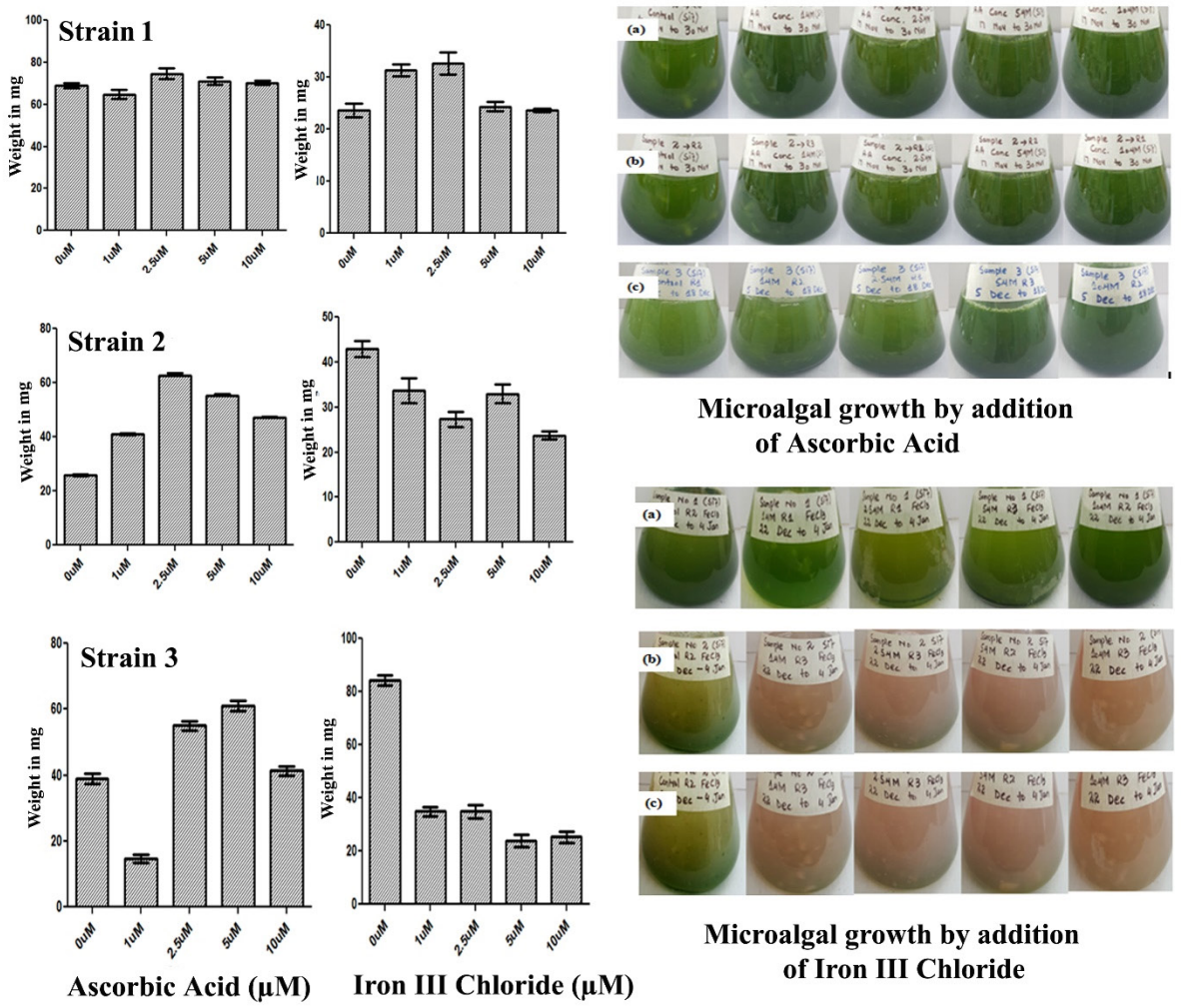
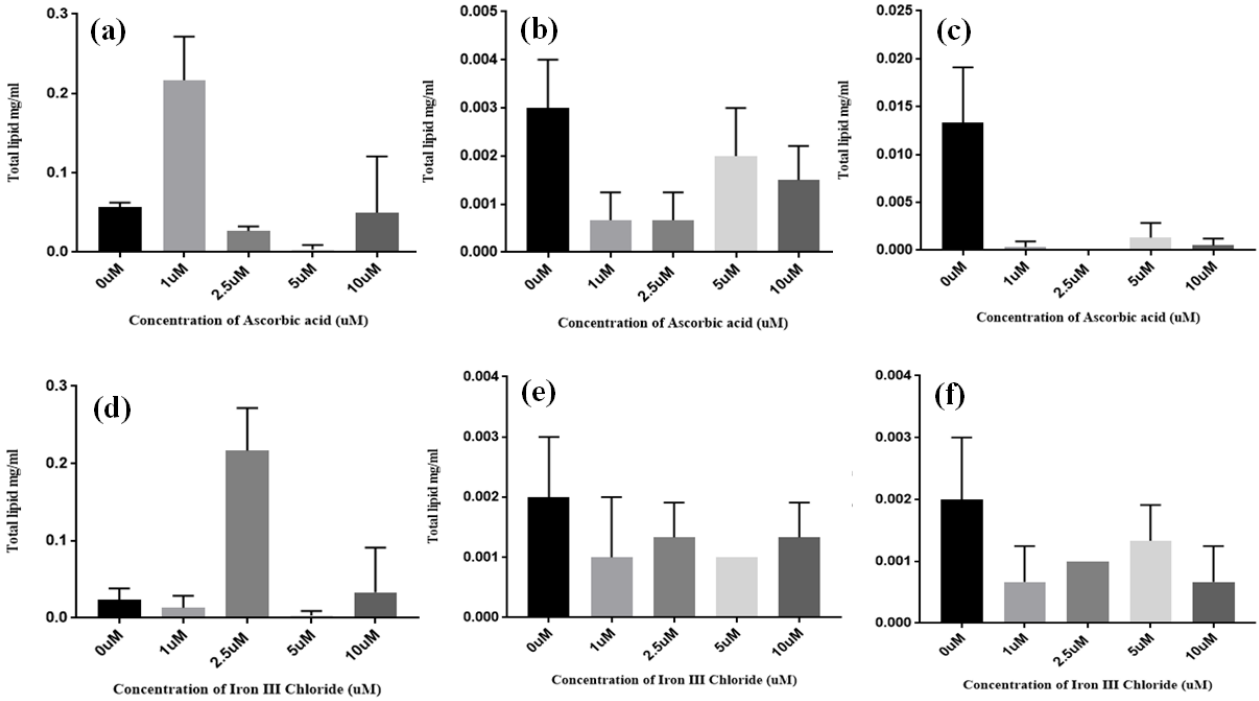


FIGURE 1. Assessment of biomass production by the addition Ascorbic acid and Iron III chloride

ASSESSMENT OF LIPID YIELD BY THE ADDITION OF CHEMICAL SUBSTRATES

Lipids extraction from the microalgal strains 3 days after reaching the stationary phase was done and the results show decreased lipid productivity as compared to biomass production. By the addition of ascorbic acid and $FeCl_3$, only the *C. sorokiniana* strain 1 showed effective results at 1 & 2.5 µM, respectively. The difference in lipid accumulation shows that growth condition and availability of carbon source play an important role in the storage of lipids (Figure 2).

Figure 2(a) represent the effect of different concentrations of Ascorbic Acid on lipid productivity '*C. sorokiniana* strain 1 (Safari Wildlife Park, Lahore)', while 2(b) represents effect of different concentrations of Ascorbic Acid on lipid productivity '*C. sorokiniana* Strain 2 (Bahria Town, Lahore)' and 2(c) represent effect of different concentrations of Ascorbic Acid on lipid productivity '*C. sorokiniana* strain 3 (Sukh Chane Society, Lahore)'. Similarly, Figure 2(d) represents the effect of different concentrations of Iron III on lipid productivity '*C. sorokiniana* strain 1 (Safari Wildlife Park, Lahore)', while 2(e) represents effect of different concentrations of Iron III on lipid productivity '*C. sorokiniana* strain 2 (Bahria Town, Lahore)' and 2(f) represents effect of different concentrations of Iron III on lipid productivity '*C. sorokiniana* strain 3 (Sukh Chane Society, Lahore)'.



The data expressed as mean ± standard deviation of the three tests (n=3). Significant differences ($p<0.05$) were observed at different concentrations of chemical substrates

FIGURE 2. Effect of different concentrations of Ascorbic Acid and Iron III Chloride on lipid productivity '*C. sorokiniana* strain 1 (Safari Wildlife Park, Lahore)' Strain 2 (Bahria Town, Lahore) and Strain 3 (Sukh Chane Society, Lahore)

FATTY ACID PROFILING

Fatty acid profiling showed a significant increase at both 1 µM ascorbic acid and 2.5 µM $FeCl_3$ concentrations, respectively. While all the other constituents were present in minor amount which includes C16:1, C16:2,

C16:3, C18:0 (Table 2).

TABLE 2. Fatty Acid Methyl Esters (FAME) profiling of '*C. sorokiniana* strain 1 (Safari Wildlife Park, Lahore)' at 1 µM ascorbic acid & 2.5 µM iron III chloride concentrations. Data were expressed as a percentage of a typical experiment

	Control	1 µM ascorbic acid	2.5 µM iron III chloride
C16:0	15.008	16.396	17.094
C16:1	5.228	5.448	5.293
C16:2	5.603	5.383	6.288
C16:3	12.682	12.667	11.893
C18:0	1.594	1.379	3.431
C18:1	13.862	14.232	12.384
C18:2	13.034	11.68	15.784
C18:3	26.164	27.622	28.748

PHYSIOCHEMICAL CHARACTERIZATION

pH results showed that all the extracted oil samples are acidic in nature and have acidic pH value of 2. The acid value obtained was different for all the strains at different concentrations (Table 3) due to hydrolytic rancidity and so the samples with higher acidic value were more rancid. According to results, the *C. sorokiniana* strain 1 was more rancid at A.A concentration of 10 and 0 µM for $FeCl_3$ *C. sorokiniana* strain 2 had highest acidic value at 0 µM of A.A while two different concentration of $FeCl_3$ (0, 10 µM) gives the same results. The third strain *C. sorokiniana* gives the highest acidic value at 2.5 µM of ascorbic acid while 0 µM for $FeCl_3$.

TABLE 3. Acid values of the three isolated novel strains at diff. concentrations of Ascorbic acid & Iron III chloride

	Ascorbic Acid			Iron III Chloride		
Concentrations (µM)	*C. sorokiniana* Strain 1	*C. sorokiniana* Strain 2	*C. sorokiniana* Strain 3	*C. sorokiniana* Strain 1	*C. sorokiniana* Strain 2	*C. sorokiniana* Strain 3
0	1.2903	1.683	1.492	1.307	1.683	1.560
1	1.122	1.309	1.307	0.931	N/A	1.201
2.5	1.492	1.122	1.851	1.122	1.30	1.019
5	1.307	1.496	1.307	1.122	N/A	1.023
10	1.683	1.496	0.931	1.22	1.683	0.855

RELATIVE DENSITY

According to our results, the *C. sorokiniana* strain 1 gives the highest relative density at the 1 µM of A.A and 5 µM of $FeCl_3$. *C. sorokiniana* strain 2 has highest relative density at 10 µM of A.A as well as $FeCl_3$. The

C. sorokiniana Strain gives the highest at 5 µM for A.A and 0 µM for $FeCl_3$ (Table 4).

TABLE 4. Relative density of the three isolated novel strains at diff. concentrations of Ascorbic acid & Iron III Chloride

	Ascorbic Acid			Iron III Chloride		
Concentrations (µM)	*C. sorokiniana* Strain 1	*C. sorokiniana* Strain 2	*C. sorokiniana* Strain 3	*C. sorokiniana* Strain 1	*C. sorokiniana* Strain 2	*C. sorokiniana* Strain 3
0	0.655	0.732	0.690	0.550	0.624	0.727
1	0.820	0.706	0.659	0.544	0.670	0.720
2.5	0.681	0.709	0.721	0.490	0.669	0.718
5	0.784	0.767	0.836	0.640	0.642	0.709
10	0.768	0.770	0.638	0.630	0.777	0.716

DISCUSSION

Microalgae have gained attention for its potential use in the 3rd generation biofuel production as they are present in all ecosystems and provide a source of environment friendly fuel production method (Srivastava & Prasad 2000). Our study showed the colonial characteristics of the three isolated microalgal cultures, identified as *Chlorella sorokiniana* (Anitha & Narayanan 2012). Different physical and chemical factors contribute in the growth of microalgae and the oil derived from it (Vermue et al. 2018). If provided with suitable conditions microalgae biomass double within 3.5 or 24 h in the exponential growth phase (Chisti 2007).

Our study mainly focused on two chemical substrates; ascorbic acid and $FeCl_3$. Ascorbic acid (vitamin C) is well-known for its nutritional importance and its contents in different microalgae species have already been reported and mean percentage values for ascorbic acid ranged from 9.4 to 700 fg/cell. Biosynthetic pathway for ascorbic acid metabolism has not been established even though it reaches millimolar concentrations in most tissues (Foyer 2017). Different mechanisms and roles about the Iron are reported like it reduces the cost of downstream processes from culturing to esterification and increases in the cell density for biofuel production (Seo et al. 2015).

The comparison of growth rate and dry mass of isolated strains gave different results at different concentrations of ascorbic acid and Iron III chloride, $FeCl_3$ (0, 1, 2.5, 5, and 10 µM) for each strain. Test for their lipid production showed that the *C. sorokiniana* strain 1 gave maximum growth at a concentration of 2.5 µM of that of ascorbic acid and $FeCl_3$ (Yao et al. 2012). *C. sorokiniana* strain 2 gave maximum growth at the same concentration of ascorbic acid but it was inhibited by the addition of $FeCl_3$. For strain 3 maximum growth was observed at a 2 & 5 µM ascorbic acid (Yao et al. 2012), while it was inhibited by the addition of $FeCl_3$ in case of 3rd strain. The reason in case of ascorbic acid is the metabolic gene ascorbate which enhance the biomass production (Zalogin 2012), triggers the metabolic genes involved in the production of biomass. Though this experiment explores the effect of iron source at a lower concentration and neutral pH, regulatory gene is still unknown because only the phenotypic involvement of this chemical is observed till now (Prommuak et al. 2013).

The lipid content profile on the 15th day after incubation indicated that microalgae grown under chemical stress have decreased lipid productivity as compared to biomass production. By the addition of ascorbic acid and $FeCl_3$, only the strain 1 shows effective results at 1 & 2.5 µM respectively (Figure 1). These variations in lipid accumulation suggest that growth conditions and carbon source availability are critical factors in lipid storage. In general, these considerations give information on changes in carbon metabolism that might affect carbon partitioning in microalgae and lead to oil buildup.

Data on lipid content from a prior study indicates that microalgae species acquire significant amounts of lipids (Sharma et al. 2018), contributing to a high oil yield (Wang et al. 2010). In previous studies (Soratana & Landis 2011), total lipid contents consist of 20-50% of the dry biomass weight and even exceed above 90% in some cases in response to different culture conditions

(Mata et al. 2010). Two microalgae spp. *Chlorococcum* sp. UMACC 112 and *Scenedesmus* sp. DM (0.28 and 0.26 g L^{-1} d^{-1}, respectively) are known for the best biomass as well as lipid producer (Rizwan et al. 2017).

These results also showed that the strains which are effective for high biomass production have no obvious relation to high lipid yield. Metabolic genes are responsible for the high biomass productivity while enhanced lipid yield depends on carbon source. Thus, for biofuel applications, it might be useful to manipulate the flow of photosynthetic carbon towards the accumulation of lipids, rather than inhibiting or enhancing the factors detrimental to growth (Griffiths & Harrison 2009).

Formation of Fatty Acid Methyl Esters (FAME) by transesterification of lipids having similar characteristics as Petro-diesel oil also a biofuel (Johnson & Alric 2017). The FAME profile of strain 1 at 1 µM ascorbic acid and 2.5 µM iron III chloride significant increase. While all the other constituents are present in minor amount which include C16:1, C16:2, C16:3, C18:0. Palmitic acid, oleic acid, linoleic acid, and linolenic acid are major constituents of the microalgae spp. cultured in growth medium treated with chemical substrates (Table 2).

Regardless, quality of oil must fulfill the criteria according to the national standards. pH is used for the quantification of acidic or alkaline nature of an oil. pH results show that all the extracted oil samples are acidic in nature and have acidic pH value of 2.

Acid value is a common parameter in the specification of oil or fat sample. It is a measure of free fatty acids (FFA) present in the oil sample and is responsible for flavors and aroma of the oil. Thus, acid value indicates the rancidity of the oil. The fatty acids with long chain carbon atoms either saturated or unsaturated experience more effects from hydrolysis then short chain fatty acids resulting in production of rancidity or different aroma. In our results, the acid value obtained was different for all the strains due to the process known as hydrolytic rancidity. Those samples with higher acidic value are more rancid (Kardash & Tur'yan 2005).

Relative density is basically the ratio of the density with respect to that of water. According to our results, the strain 1 gives the highest relative density at the 1 µM Concentration of ascorbic acid and 5 µM of $FeCl_3$. Strain 2 has highest relative density at 10 µM of ascorbic acid as well as $FeCl_3$. While the Strain 3 gives the highest at 5 µM for ascorbic acid and 0 µM in case of $FeCl_3$. It is not mandatory that all those samples which are source of good quality oil are also used for the production of biofuels, the reason behind this is the fatty acid profiling of the lipids.

An open raceway pond is less expensive than photo bioreactors, easier to clean and maintain, suitable for modest energy inputs and helpful where non-agricultural areas are available. Because of its cost-effectiveness, an open raceway pond should be used. However, the continued exploitation of the opportunities offered by microalgae still faces some challenges. The cost of producing biodiesel is still quite expensive due to small-scale production of algae biomass in the lab. However, if energy authorities give cost-effective incentives and subsidies, biodiesel production from the best growing algae might surge. On the technological perspective, the crucial infrastructure needed for production of biofuel would have to be established (Abdo et al. 2020; Bhardwaj et al. 2020; Davis et al. 2011; Giwa et al. 2018; Narala et al. 2016). In comparison to open raceway ponds the cost effectiveness might face challenges due to small scale production and utilizing different biochemical processes. In current study, *C. sorokiniana* strain 1 (Safari Wildlife Park, Lahore) is concluded for the biofuel production in our local vicinity.

Conclusion

Current study was aimed to isolate local strains of microalgae to check their potential towards the production of biofuel by the addition of different chemical substrates. As substrates, five different concentrations of ascorbic acid and iron (III) chloride (0, 1, 2.5, 5, and 10 µM) are employed. Microscopic examination indicated that the samples are from the genus Chlorella, and DNA analysis confirmed that the samples are *C. sorokiniana.* Among all ascorbic acid concentrations tested, 2.5M was more efficient against *C. sorokiniana* strains 1 (Safari Wildlife Park, Lahore) and 2 (Bahria Town, Lahore), whereas *C. sorokinian* strain 3 (SukhChane Society, Lahore) reacted at 2.5 and 5M in terms of biomass production. The growth of *C. sorokiniana* strains 2 and 3 is suppressed by $FeCl_3$ (2.5M). At 1 and 2.5M of ascorbic acid and $FeCl_3$, respectively, only the *C. sorokiniana* strain 1 displays effective results, according to lipid content analyses. Fatty acid profiling was performed prior to those concentrations that produce substantial lipid synthesis findings. According to the findings, *C. sorokiniana* strain 1 can be used as a source of alpha-linolenic acid, which is a key component in the manufacture of biofuels. It is inferred that *C. sorokiniana* strain 1 can be used to produce environmentally benign biofuels based on our findings.

ACKNOWLEDGEMENTS

Authors acknowledge Higher Education Commission Pakistan for providing funding support to Safdar Abbas (2BM2-115) under Indigenous PhD Fellowship (Phase II). Authors also acknowledge the laboratory staff of Department of Biochemistry Faculty of Biological Sciences Quaid-i-Azam University Islamabad Pakistan and Institute of Molecular Biology and Biotechnology University of Lahore.

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*Corresponding author; email: snq28@yahoo.com