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Study on Biomass and Lipid Production by Novel Microalga *Scenedesmus quadricauda* KDPSC2 Utilizing Ultrasonic Pretreated Leather Industry Effluent: A Low-Cost Medium for Biodiesel Production

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ABSTRACT

Microalgae biomass are considered as a sole prime feedstock for third generation biofuels, due to they possess many advantages than the feedstock of land-based biomass. In this study, biomass and lipid producing potential novel isolate *Scenedesmus quadricauda* KDPSC2 by utilizing ultrasonic pretreated leather industry effluent (LIE) as sole medium was examined. The optimization of LIE pretreatment conditions was performed by response surface methodology using the design expert software-Minitab12. The maximum biomass of 49.21 ±0.31 gL^{-1} and lipids content of 36.11±0.32 % DW was obtained from LIE pretreated by ultrasonically at 0.35WmL^{-1} for 20 min. The extracted lipid contained highest amount of 82.21±0.14% triacylglycerols (TAGs) that serve as material for biofuels production. Hence, the results of present investigation suggest the possibility of utilizing ultrasonic pretreated LIE as a low cost culture medium for biodiesel production from microalgae. *S. quadricauda* KDPSC2 was also proved to be a potential strain for biodiesel production from LIE.

KEYWORDS: Biomass, leather industry effluent, Lipid, *Scenedesmus quadricauda* Ultrasonic

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INTRODUCTION

The energy requirement is gradually increasing due to rapid development in industrialization and population. The fast depletion of fuel energy and its adverse effects on the environment have strongly prompted the researchers to find suitable alternative renewable resources for fossil fuels. In this juncture, biodiesel could be a promising alternative to fossil fuels due to its environmental benefits [1-2]. The production of biodiesel from various edible oil sources such as corn, palm, soybean and rapeseed oils has gained remarkable attention due to better lubrication, low toxicity, eco-friendly, sufficient energy density, suitability with currently available diesel engines, low emission profile and sustainability [1,3-5]. However, these raw materials are increasing the cost of production of biodiesel as they are a main part of the food and their mass cultivation is limited due to the constant depletion of farmland and climate change[6].

Using oleaginous microorganisms is an alternative strategy for low cost biodiesel production. Many studies have been focused on oleaginous microorganisms including bacteria, micoralgae and yeasts with high concentration (>20 %) of lipid content as an alternative source for biodiesel production owing to similarity with vegetable oil fatty acids [7-8]. However, the economic feasibility of these sources oils remains uncertain due to their cultivation cost, which accounts for 40-80% of overall biodiesel production cost [9-10]. To accomplish economic and sustainable biodiesel production from the oleaginous microorganisms, they should be cultivated using low-cost substrates. Organic particulates contain organic wastes may be ideal and inexpensive substrates for microbial biodiesel production. However, some microbial strains may not be able to utilize the organic wastes because its chemical compositions affect growth and lipid production. Therefore, the characteristics of each organic waste and each microbial strain should be considered simultaneously [11].

Among the oleaginous microbes, microalgae are a promising third generation feedstock for biofuel production and lipid production due to its rapid growth rate, higher biomass production, rich oil content, strong ability to sequester carbon dioxide and rapid conversion of CO^2 into methane or hydrogen. In addition, the intensive production of microalgae is a viable technology for wastewater treatment and as a source of sustainable biomass to meet the ever-increasing demand for food and energy [12]. However, using artificial media for large-scale production of microalgae biomass is economically nonviable. Hence, the utilization of wastewater for cultivation of microalgae is a cost-effective option because they contain more organic and inorganic compounds.

The leather industry effluents (LIE) are highly complex because of the presence of various chemical pollutants. Therefore, the LIE have been identified as main causes of soil and water pollution in all over the world. They Bioremediation technology to treat LIE have gained considerable attention, as it is more favorable and economical relative as compared to other

technologies [13]. Microbes such as bacteria [14], fungi [15], microalgae[16], and yeasts[17] are capable in removing the pollutants significantly by utilizing them as nutrient source under the aerobic or anaerobic condition[18]. However, the excess concentration of organic maters are limited the growth of microbes. Therefore, prior to use, an appropriate pretreatment need to convert the complex organic compounds into simple one for efficiently utilize by microbes. To facilitate this, various sole and combined disintegration techniques such as thermal, thermo-chemical, ultrasonic and thermo-chemo-sonic digestion have been applied with high-energy inputs, as reported in the literature. Application of these technology, leads to the decomposition of many organic compounds during the process [19-20].

Ultrasonic intensification can be used as a pretreatment prior to the treatment of industry effluent by microalgae for the production of biodiesel. Because they are low operating cost technique than other enhancing treatment options. Besides simplicity of operation, modest power requirements and does not require sophisticated equipment reformats or intensive technical training for utilization[21-22]. Highest removal of organic pollutants and nitrogen from low ultrasound pretreated wastewater was achieved by variety of biological activities[23-25]. But either too low power or too high level of ultrasonic led to decrease of biological activity and degradation rate[26].

Freshwater microalgae with high contents of lipids as well as a high growth with nutrient removal from wastewater have yet to be exploited for biodiesel production, and isolation and characterization of potential microalgae for more efficient oil production remain the focus of continuing research[27]. From the literature, it was observed, there was no report on isolating novel oleaginous microalgae from Mamandur freshwater Lake until now, and also very few studies were reported on the production of lipids for biodiesel production using unsterilized pre-treated LIE as a nutrient medium. Therefore, the present study main objectives are to (i) isolate oleaginous microalgae from freshwater Lake (ii) screen the potential oleaginous microalgae by test their consistency of growth in 10% raw LIE (iii) identify the selected potential strain using molecular techniques; (iv) optimize the process parameters for ultrasonic pre-treatment of LIE and use as sloe culture medium for batch cultivation of selected microalgae strain (iv) characterize the lipids extracted from selected strain grown in the pretreated LIE.

MATERIALS AND METHODS

Materials

In this study, various chemicals with highest purity or analytical grade were purchased from Himedia chemicals (Mumbai, India), Sigmae-Aldrich (Bommasandra, India) and Merck Chemicals Ltd.,(Mumbai, India) and used for preparation of media and reagents.

Sample collection

For the isolation of oleaginous microalgae (phytoplankton), the water samples were collected from four different sites along the waterfront of Mamandur Lake (12°45'14" ˚N and 79°39'24"˚E) which is situated in Cheyyar Taluk's, Tiruvannamalai District, Tamil Nadu, India. All sites samples were collected in 100 mL sterilized screw caped bottles and maintained at refrigerated condition while transferring to laboratory.

Isolation and screening the potential oleaginous microalgae

All the four sites samples were pooled and eliminated the bacterial contamination by the antibiotic treatment[28]. Then the sample was serial diluted until 10^{-5} dilution. Dilutions 10^{-3} to 10^{-5} were chosen and 100 µL of the each dilution samples were spread evenly on petri dishes (100 X 15 mm) containing approximately 40 mL agarized (20 gL^{-1} agar) Bold's Basal medium[29], AA medium[30], F/2 medium[31], and modified Chu13 medium[32]. Inoculated plates were incubated at 25±2°C under light intensity of 33 µE $m^{-2}s^{-1}$ with 12:12 h light:dark cycles until the colonies were appeared. Then, the colonies were streaked on additional sets of nutrient media plates and this was repeated until getting the axenic culture. The culture purity was examined by regular observation under light microscope (Olympus CX21i-LED. Binocular Version).

All the isolated microalgae were screened for their ability to grow in 10% untreated leather industry effluent and produce lipids by incubating at 25 ± 1 °C under 33 µE $m^{-2}s^{-1}$ light intensity with 12:12 h light and dark cycles for 10 days. The effluent pH was adjusted to pH 6.8±0.2. On the 11 day the cultures were withdrawn and used for estimation of biomass and lipid. Screening experiments were carried out in triplicate for each microalgal strain. All values are represented as mean ± SD of three replications. The maximum biomass and lipid producing strain was selected and used for further investigation.

Cell biomass estimation

Cultures were withdrawn and cells were harvested by centrifuged at 14,000 rpm for 15 min. The cell biomass was estimated by gravimetrically method[33]. All values are represented as mean ± SD of three replications.

Molecular level identification of selected isolate

The selected isolate was identified by genes amplification by PCR using the published primers for chloroplast-encoded rbcL (F:5'GAT GCA AAC TAC ACA ATT AAA GAT ACT G3' & R:5'ATT TTG TTC GTT TGT TAA ATC CG3'),18S rDNA (F:5CAA GTT TCT GCC CTA TCA GCT3' & R:5'GCT TTC GCA GTA GTT CGT CTT3') and ITS (Internal transcribed spacer) (F:5'GTC GCA CCT ACC GAT TGA3' & R:5'CGG GTA GCC TTG CTT GAT3')[34]. The PCR

product was sequenced by according to the manufacturer's protocol using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems 3730XL) and then the sequence was deposited in GenBank. The obtained nucleotide sequence was used for similarity search and the phylogenetic tree was build using MEGA7 (Molecular Evolutionary Genetics Analysis)[35] version 7.

Collection and characterization of leather industry effluent

Effluent was collected from Ranipet SIPCO finished leather effluent treatment company Ltd., Ranipet, Vellour, Tamil Nadu, India using sterilized 5L bottles, brought to the laboratory and stored at room temperature until used for analysis. Concentration of dissolved organic matter (DOM) was estimated before and after ultrasonic pre- teratment of effluent. DOM was estimated by Khan et al., method[36.]

Optimization of ultrasonic pre-treatment of LIE

Ultrasonic pretreatment of LIE was carried out by Sonicator (Lark Innovative Fine Teknowledge, Chennai, India) with a probe of 2mm in 250 mL stainless steel beaker containing 150 mL of 55% diluted LIE. A 2^2 Central Composite Design (CCD) was used to optimize the ultrasonic parameters including ultrasound power density (UPD) range from 0.2 to 0.5 WmL^{-1} and exposure time (10 to 30 min). A 13 experiments were designed and formulated for these two factors using the design expert software-Minitab 12. During sonication, the increase of wastewater temperature was controlled by a water-cooling bath gradually and temperature of the samples did not exceed 35^oC during the experiments. One sample without exposure was served as a control. Experimental biomass concentrations were obtained using CCD was statistically analyzed and fit into second order polynomial model. The predicted percentage of biomass concentration and optimal levels of the variables were found by solving the model for maximization of response using the MINITAB 12 software.

Cultivation of selected microalga strain in ultrasonic pretreated LIE

Selected isolate was cultivated in 250 mL Erlenmeyer flasks contained 100 mL of ultrasonic pretreated LIE and sealed with cotton. Prior to inoculate, the pretreated effluent medium pH was adjusted to 7.0 using 1N NaOH or HCl. Microalgal seed inoculum was added to flasks at a fixed volume (10% v/v). Then the flasks were kept in a thermo controlled shaking incubator at 100 rpm and 25±1°C with illuminated using fluorescent light (33µmol photons m^{-2} s^{-1}) with a 12:12 h light : dark cycle for 10 days.

Analysis of bacterial population in pretreated effluent

Before and after the pretreatment, bacterial population of LIE sample was estimated by plate counting. Aseptically, 1mL of diluted sample was spread on nutrient broth (NB) agar plate and

incubated at 37°C for 24 hours. At the end of the incubation period, petri plates containing bacterial colonies were counted by digital colony counter. The colony forming unit (cfu) arrived by the following formula,

CFU/ml = (no. of colonies x dilution factor) / volume of culture plate

Extraction and Gas Chromatography analysis of lipids

The total lipid content was extracted and determined by Blight and Dyer method[37]. The lipid profiles were analyzed using Gas Chromatography by Widjaja et al., method[38].

Statistical analysis

The results of all the experiments were presented as the mean ± standard deviation (SD) values of three independent replicates. The one-way analysis of variance (ANOVA) also done for the obtained data using MINITAB 12 software at the significant level of $p \leq 0.05$.

RESULTS AND DISCUSSION

Isolation and Screening of Biomass Producing Microalgae

The very first step of this study was focused on isolating maximum biomass producing freshwater microalgal utilizing LIE and accumulating high lipid content. For this purpose, ancient lake water was used as the source for isolation with four different medium viz Bold's Basal medium, AA medium, F/2 medium and modified Chu13 medium. A total of 12 strains were isolated by serial dilution, pour plating and plating techniques. All the isolates were designated as KDPSC1 to KDPSC12 serially. When all the 12 isolates were individually subjected to screen by cultivating in 10% raw LIE for 10 days, only the isolate KDPSC2 was showed maximum cell biomass of 27.11±0.75 gL^{-1} and lipid content of 15.36±0.01 %. Then, it was used for further investigation. This result supports the fact that the biomass production as well as accumulation of lipid content can change tremendously among the species [39].

Identification of Microalga Strain KDPSC2

Molecular level identification of strain KDPSC2 was done by 18S rDNA sequence analysis. The gene encoding of 18S rDNA was amplified by PCR technique and found that the amplified product size as ~974bp. For sequence similarity search, the selected microalga strain sequences was compared with closely related microalgae sequences existing in the GenBank database. The distance matrix was generated with length of 1000 replicates suing Jukes-Cantor method [41]. Then the phylogenetic tree was constructed with 29 related nucleotide sequences by Neighbor-Joining method[40] using the software MEGA version 7. In the constructed phyolgenetic observed that the isolate KDPSC2 is nearly 100% closed to the strain *Scenedesmus quadricauda* BUM11012 (accession number: KC218486) (Figure 1). The phylogenetic analysis information revealed that the

isolate KDPSC2 was corresponding to *Scenedesmus quadricauda*. Hence, this strain sequence was submitted to GenBank as *Scenedesmus quadricauda* strain KDPSC2, and the assigned GenBank accession number was MH238504.

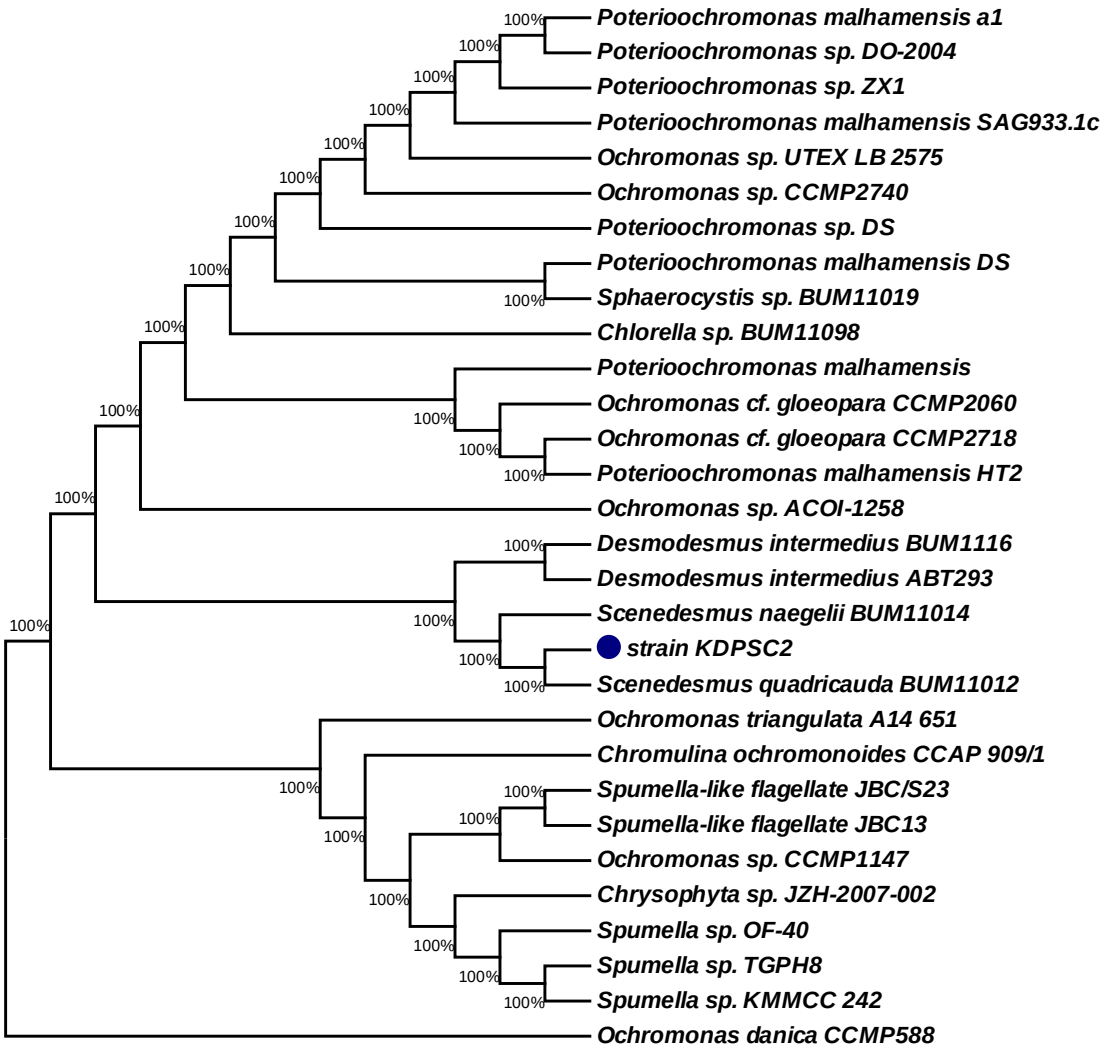


Fig. 2 The phylogenetic tree of *S. quadricauda* strain KDPSC2, (accession number: MH238504) was constructed using neighbor joining method. The marker represents the 18S rDNA gene sequence of strain KDPSC2.

Optimization of Ultrasonic Pretreatment of LIE for S. quadricauda KDPSC2 Cultivation

High lipid containing microbial biomass could be considered as promise feedstock for biodiesel production[2]. Hence, the sustainable microbial biomass production for commercialization of biodiesel is most important because microbial growth rate, biomass productivity, and concentration of lipids are fundamental parameters [42]. In this study, LIE was pretreated ultrasonically and then used for *S. quadricauda* KDPSC2 cultivation. The LIE pretreatment optimization was studied using CCD of RSM. In order to determine optimum UPD and exposure time for pretreatment of LIE, biomass

was considered as an indicator for parameters optimization. Analysis using a set of 13 experiments was done. All the experiments were carried out in duplicate. The design matrix included two independent variables of UPD (X_1) and exposure time (X_2), and the predicted and experimental values of response (Biomass production) of *S. quadricauda* KDPSC2 were shown in Table 1. The CCD results was analyzed using coded units (Table 2). The model expressed by the following regression equations (1) represent biomass production (Y) as a function of UPD (X_1) and exposure time (X_2).

$$Y_{CODED} = 49.20 + 1.845X_1 + 3.52X_2 - 9.36X_1^2 - 4.37X_2^2 + 1.49X_1X_2 \quad \text{-----(1)}$$

where Y is the biomass production(gL^{-1}), X_1 and X_2 are the coded value of UPD and exposure time respectively.

The results of the RSM obtained were plotted as the three dimensional response surface curves to find out the optimum level of UPD and exposure time for LIE pretreatment and make it as a best medium for maximum biomass and lipid production by *S. quadricauda* KDPSC2. From the surface plot (Figure 1) the UPD 0.35WmL^{-1} and exposure time 20 min found as the optimum level for pretreatment of LIE, because in which the maximum biomass of 49.21±0.31 gL^{-1} and lipid of 36.11±0.32% was obtained by *S. quadricauda* KDPSC2. This may be because of the availability high dissolved nutrient contents, which were liberated from LIE during the pretreatment at these conditions.

Significance and adequacy of second-order polynomial equation was examined by ANOVA (Table. 3). In this study, the coefficient of determination (R^2) value was 0.993, which is closer to 1. Therefore, the correlation is better between the experimental and predicted values by the second order polynomial model [43]. The adjusted R^2 value 0.989 was also very close to R^2 value. Hence, the model is well fitted to represent the effect of variables (UPD and exposure time) on ultrasonic pretreatment using RSM.

Table No. 1: Experimental and predicted response obtained using RSM for ultrasonic pretreated LIE

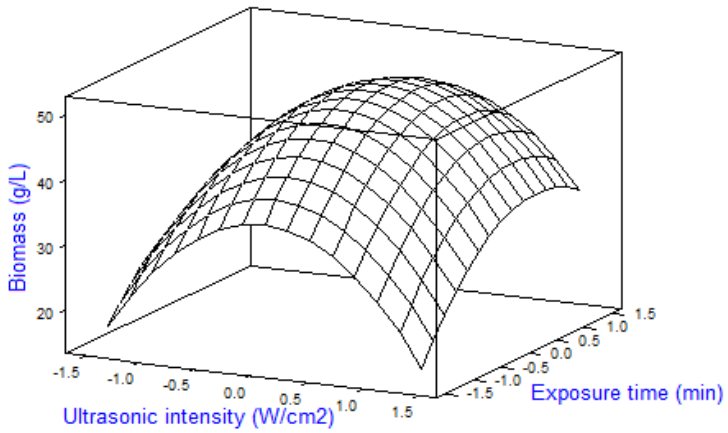
Run Order	Ultrasound Power density (WmL^{-1})	Exposure time (min.)	Biomass concentration (gL^{-1})	
			Experimental	Predicted
1	0.20	10.00	31.21	31.59
2	0.50	10.00	31.21	32.29
3	0.20	30.00	35.23	35.64
4	0.50	30.00	41.21	42.32
5	0.13	20.00	28.11	27.85
6	0.56	20.00	34.32	33.07
7	0.35	5.85	36.21	35.48
8	0.35	34.14	46.21	45.44
9	0.35	20.00	49.21	49.204
10	0.35	20.00	49.20	49.204
11	0.35	20.00	49.21	49.204
12	0.35	20.00	49.20	49.204
13	0.35	20.00	49.20	49.204
Control -55% diluted effluent		-	24.14	

Table No. 2: Estimated regression coefficients of second order polynomial model for ultrasonic pretreated LIE

Variables	Estimated Coefficients	t-value	p-value
Model	49.20	124.47	<0.001*
X_1	1.845	5.90	<0.001*
X_2	3.52	11.26	0.002*
X_1^2	-9.36	-27.95	<0.001*
X_2^2	-4.37	-13.04	0.002*
X_1X_2	1.49	3.38	0.012*

R-Sq = 99.3% R-Sq(adj) = 98.9% * Significant

Figure 2. Response surface plot for biomass production of *S. quadricauda* KDPSC2 cultivated in optimized ultrasonic pretreated LIE



The P-values were small which indicate the higher significance of the corresponding variable. In the present study, the model obtained was significant ($P < 0.05$) suggesting the

ultrasonic pretreatment variables such as UPD and exposure time could play a synergistic role in changing the complex characteristics of LIE. The statistical results showed that the ultrasonic pretreatment is a significant factor in prior utilization of LIE for biomass and lipid production by *S. quadricauda* KDPSC2 ($p= 0.012$, $p< 0.05$). This can significantly enhance the growth of *S. quadricauda* KDPSC2. A 19% increased methane gas was produced from ultrasonic pretreated meat wastewater by anaerobic processes [44].

Table No. 3: Analysis of variance (ANOVA) results of optimization of ultrasonic pretreatment of leather industry effluent for *S. quadricauda* KDPSC2 cultivation

Source	Degree of freedom (DF)	Sum of Squares (SS)	Mean Square (MS)	F-value	p-value
Regression	5	816.125	163.225	208.93	<0.001
Linear	2	126.379	63.189	80.88	<0.005
Square	2	680.806	340.403	435.73	<0.001
Interaction	1	8.940	8.940	11.44	0.012
Residual Error	7	5.469	0.781		
Lack of fit	3	5.469	1.823	*	*
Pure Error	4	0.000	0.000		
Total	12	821.594			

Bacterial Growth in Pretreated LIE

A 61 bacterial colonies were observed in untreated LIE. Whereas no single bacterial colony was observed in the LIE after ultrasonic pretreated at 0.35WmL^{-1} for 20 min. Ultrasound can be used as a disinfection to reduce the bacterial population in wastewater because the acoustic cavitation phenomenon lyse bacterial cells [45-46].

Analysis of DOM

The sonication at 0.35 WmL^{-1} for 20 min was increased the DOM concentration from 11.01±0.02 mgL^{-1} to 34.21±0.31 mgL^{-1} in LIE. This might be the consequence of ultrasound promotion. During the ultrasonic treatment process, the microbial cells and extracellular polymeric substances were thoroughly disrupted by acoustic cavitation phenomenon[47]. Further, these two components were broken into soluble organic matters. Utilization of more soluble organic matters in the fermentation broth can be obtained the greater amount of desired products [48].

Lipid Analysis

The profile of lipid extracted from biomass of *S. quadricauda* KDPSC2 was analyzed by Gas Chromatography. The lipid extracted contains ~91% triacylglycerols (TAGs), ~9% free fatty acid and trace levels of charged glycerolipids (CGLs). Therefore, this lipid can be used as a feedstock for renewable, non-toxic and biodegradable biodiesel production by transesterification. The transesterification depends on the amount of hydrocarbons and triglycerides present in the lipid

content of biomass[49]. The lipid profile of *S. quadricauda* KDPSC2 cultivated in the pretreated LIE was reasonably similar to the lipids of different oleaginous microalgae species[50].

CONCLUSIONS

The novel microalga *S. quadricauda* KDPSC2 isolated from ancient freshwater lake was proved as a potential candidate for utilization of ultrasonic pretreated LIE as a low-cost medium for production of biomass and higher amount of TGAs. The model developed using RSM for optimizing the pretreatment of LIE was efficient in predicting experimental independent variables. By the optimization of LIE pretreatment found that the low UPD ($0.35 WmL^{-1}$) could effectively promotes the effluent reduction for the growth of *S. quadricauda* KDPSC2 and lipid accumulation. Therefore, the pretreatment of LIE with low ultrasonic power density, prior to use, as media for cultivation of oleaginous microorganisms was a promising approach in the future applications of biofuel production. Besides, the lipids derived from the ultrasonic pretreated LIE grown *S. quadricauda* KDPSC2 could be a promising feedstock for biodiesel production and various industrial applications.

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