

Ultrasound assisted enhanced extraction of lutein (β , ϵ -carotene-3, 3'-diol) from Microalga (*Chlorella pyrenoidosa*) grown in wastewater: Optimization through Response Surface Methodology

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Abstract

One of the fundamental carotenoid compounds available in microalga *Chlorella pyrenoidosa* is lutein, which is a commercially valuable antioxidant compound. The extraction of lutein from *C. pyrenoidosa* was studied in detail under various operating conditions. Acid pretreatment was done using 10% HCl. To analyze the effectiveness of ultrasound based extraction, experiments were carried out in presence and absence of ultrasonication. The results showed increased lutein yields in presence of ultrasonication. Scanning electron micrographs of pretreated and ultrasonicated algae cells showed increased surface area due to formation of porous surface. There were no significant contrasts in antioxidant activity of lutein between the samples obtained from ultrasound and acid treatment. Furthermore, to optimize the operating parameters like concentration of HCl, ultrasound exposure time and microalga biomass concentration, response surface methodology (RSM) design was applied. From the experimental results the optimum values derived are incubation time 41.34 min, HCl concentration 15.63 % and biomass concentration 309.21 g/L for a yield of 3.5 mg of lutein per gram of algae. Hence, ultrasound extraction with acid treatment is the most efficient technique for the extraction of microalgal lutein.

Keywords: *C. pyrenoidosa*; lutein; optimization; ultrasound; anti-oxidant

1. Introduction

Microalgae are photosynthetic organisms that grip nitrogen (N) and phosphorus (P) in the midst of their improvement and ends up in biomass yield. This generated biomass can be changed over into a significant product through appropriate methodology (Ometto *et al.*, 2014). All the above, the freshwater microalgae bends towards the N and P concentrations level in their biomass in association with environment in the wastewater (Beuckels *et al.* 2015; Choi *et al.*, 2015). Microalgae are the

established commercial sources of high-regard chemicals, for instance, β -carotene, astaxanthin, docosahexaenoic acid, phycobilin pigments and algal concentrates for use in beauty care products. Microalgae are in like manner continuously having an impact in cosmaceuticals, nutraceuticals and food supplements. Over the latest couple of years, there has been energized excitement for microalgae as commercial sources of these and other high-valued products, driven mostly by the endeavors to grow industrially feasible biofuels from microalgae (Borowitzka, 2013).

Municipal solid waste (MSW) management is the major environmental concern and challenging process worldwide (Bogiatzidis and Komilis, 2016). In recent years, with increased global concern over energy demand and greenhouse gas accumulation, global countries have turned their efforts towards renewable energy from wastewater. Studies on anaerobic process, with high loading rate, methane gas production and less nutrients, were reported (Diamantis and Aivasidis, 2010; Blika *et al.*, 2009; Vlassis, 2012).

Lutein, one of the essential xanthophyll components assuming an imperative part in preventing or enhancing the impacts of various degenerative human diseases, for example, chronic diseases, age related macular degeneration. Due its far reaching utilize the worldwide lutein market has developed essentially in the current years with US offers of over \$150 million every year (Fernandez *et al.*, 2015). Ultrasound extraction has been applied to extract the components from plant and animal materials, for example, oil, protein, polyphenolics, and pigments. The yield of carotenoids extracted from *Dunaliella salina* by ultrasound was higher than that by supercritical liquid extraction (Macías *et al.*, 2008). The yield of lutein from *C. vulgaris* by ultrasound was the highest when compared with and maceration, soxhlet extraction, and pressurized fluid extraction (Cha *et al.*, 2010).

In this study, ultrasound assisted lutein extraction with/without acid pretreatment conditions from the microalga *C. pyrenoidosa* were optimized by response surface methodology (RSM). Moreover, the effect of ultrasound with/without acid pretreatment on the morphology of the microalgal cell, and the antioxidant activities of lutein were also determined.

2. Materials and methods

2.1. Wastewater

The wastewater utilized for the study was obtained from the wastewater treatment plant at Sri Sivasubramaniya

Nadar College of Engineering, Chennai, Tamilnadu. Before the experiments, pretreatment of wastewater is done by removing large solid particles by using filter cloth (Kimberly-Clark) and autoclaved at 121 °C for 15 min. After that chilled down to room temperature, and then kept undisturbed for settling of dissolved solids at 4 °C. After couple of days the supernatant is taken up for the further analyses. This wastewater is utilized for the development of microalgae; the nitrogen (N), phosphorus (P), and chemical oxygen demand (COD) were resolved utilizing the protocol specified for each test (Hach, 2008). The initial characteristics of the wastewater are seen in Table 1.

Table 1. RSM design for ultrasonic extraction.

Run order	Exposure Time (Min)	HCL Concentration (%)	Biomass Concentration (g/L)	Lutein Yield (mg/g) Experimental value	Lutein Yield (mg/g) Predicted value
1	30	10	200	2.73	2.62416
2	15	15	100	2.08	2.11091
3	45	5	100	1.77	1.68952
4	15	5	100	2.04	1.98411
5	30	10	200	2.83	2.62416
6	30	10	31.821	1.71	1.77085
7	45	15	300	3.41	3.44517
8	45	5	300	2.83	2.77837
9	4.7731	10	200	2.04	2.01351
10	15	5	300	2.15	2.14296
11	30	1.591	200	1.97	2.07598
12	30	10	368.179	3.23	3.19845
13	30	18.409	200	2.82	2.74332
14	30	10	200	2.52	2.62416
15	30	10	200	2.48	2.62416
16	45	15	100	1.92	1.90632
17	55.2269	10	200	2.32	2.37579
18	15	15	300	2.66	2.71976
19	30	10	200	2.56	2.62416
20	30	10	200	2.63	2.62416

2.2. Microalgae cultivation

C. pyrenoidosa was cultivated mixotrophically utilizing blend of wastewater and f/2 medium [9]. The algae culture was at that point acclimatized to develop in wastewater. For better development, the blend contains around 80% (by volume) of wastewater and 20% of f/2 medium. The microalgae was developed in a photo bioreactor (Fig.1) of 60 L capacity, agitated bubbling air (5% carbon dioxide) under 5000 lux illuminated white fluorescent bulb for 12:12 h light and dark condition for 15 days. The biomass was monitored and harvested via auto-flocculation and filtering process. Then the micro algal paste was rinsed with distilled water to remove residual salts and oven dried.

2.3. Initial trials for lutein extraction

Initial trials with various ranges of Biomass concentration, HCl % and Incubation time were performed for lutein extraction. Pre-RSM extraction trials for incubation time, biomass concentration, and percentage HCl were carried out in the following ranges: 15 min–45min (increments of 15 min), Biomass concentration 0.5– 3.0g (increments of

0.5), and 5–15 % HCl (increments of 5 %). Accordingly, then, these ranges were utilized as the upper and lower limits for the RSM design of experiment.

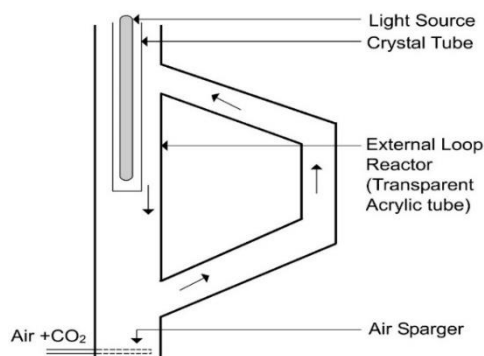


Figure 1. Photo bioreactor

2.4. Extraction

2.4.1. Ultrasound extraction

Ultrasound extraction was performed in an ultrasonic generator (VCX750, Sonic and Materials Inc, Newtown, USA) with settled recurrence of 35 kHz and constrain of 55 W/cm². Two grams of *C. pyrenoidosa* with 90% ethanol was taken in a conical flask, and afterward the jar was held in the ultrasonic tank for extraction at different incubation times and HCL %. Optimization of the ultrasound extraction condition was refined by using the response surface method (RSM) with a central composite design (CCD) (Deenu *et al.* 2013). Three distinct variables (Incubation time, HCL % and biomass concentration) were used at three equidistant levels (- 1, 0, and +1) (Table 1). The concentrate was filtered through Whatmann No. 1 paper lastly stored at 4°C for further examination.

2.4.2. Acid pretreatment

Acid pretreatment of *C. pyrenoidosa* was performed by incubating two grams of microalgae in a 100 mL of 10% HCL, and after that incubated at 50 °C or overnight. The improvement of the acid pretreatment condition was controlled by response surface system (RSM) with a central composite design (CCD).

2.4.3. Ultrasound extraction after acid pretreatment

Fresh *C. pyrenoidosa* (two grams) was incubated with 10% HCL for 30mins and afterward sonicated at fixed frequency and intensity (35 kHz, 55 W/cm²). The ultrasound extraction after acid pretreatment was optimized by reaction surface methodology (RSM), with a central composite design (CCD). The concentrate was filtered through Whatmann No. 1 paper and after that kept at 4 °C without light for further examination.

2.5. Purification of lutein

The *C. pyrenoidosa* concentrate was saponified by including 6% KOH (w/v) at 50 °C for 30 min. The blend was then subjected to rotational evaporator and the dry solid acquired was dissolved in refined water (200mL) and 200 mL ethyl acetate is added to it. At that point subjected to rotating evaporator and re-dissolved in 5 mL of acetone. The acetone solution (2 mL) was stacked on a silica gel fragment (2.0 × 60.0 cm), and after that eluted with 150 mL of hexane, followed by 300 mL of hexane-acetone (7:3 v/v). Further the separated portion is dried and stored at 20 °C.

2.6. HPLC analysis of Lutein

The filtrate of 20 µL was infused into the HPLC system (Perkin Elmer, USA) Separation was performed using a C18 column (5 mm, 150 mm × 46) at 30 °C. The mobile phase

comprised of methanol/acetonitrile (90:10 V/V). The aggregates were eluted at a stream rate of 1 ml/min and the lutein content was detected utilizing an UV visible spectrophotometer (Model V630, JASCO Analytical Instruments, Japan) by measuring absorbance at the wavelength range of 300-600 nm. The maximal absorbance (445 nm) was taken up for evaluation of lutein concentrates.

2.7. Microalgal cell morphology

Microalgal cell morphology was considered on the dried cell procured by each extraction procedure (ultrasound extraction, acid treatment and ultrasound extraction with acid pretreatment). The surface characteristics were recorded using Scanning Electron Microscope (Hitachi S-3400 Model, Tokyo, Japan) at the extent of 30 ~ 200 kV voltage.

2.8. Experimental design and optimization by central composite design (CCD)

Perfect condition for the lutein extraction from the microalgae was analyzed by response surface methodology (RSM) and the imperative class of second- order design called central composite design (CCD) was used for the examination. Advance studies were carried out by considering the effect of three variables likely HCL %, incubation time and biomass concentrations. To depict the effect of these three components, a full factorial CCD inciting 20 sets of tests were performed. The test outline and results of the CCD were appeared in Table 1. The conduct of the system is clarified by the next second-order polynomial model:

$$Y = b_0 + \sum_{i=1}^3 B_i X_i + \sum_{i=1}^3 B_{ii} X_i^2 + \sum_{i=1}^3 \sum_{j=i+1}^3 B_{ij} X_i X_j \quad (1)$$

Variance analysis was conducted and the binary quadratic condition (Eq. 1) was developed where Y is the anticipated response, b_0 is the intercept, B_i is the linear coefficient, B_{ij} are the quadratic coefficients, and X_i and X_j are the levels of the independent variables. Experimental data generated from the central composite design were fitted to a second-order polynomial regression model were fitted to a second-order polynomial predicted regression model (Eq. 2) where Y is the anticipated response (here, lutein yield), and X_1 (incubation time), X_2 (HCL %) and X_3 (biomass concentration) are the coded estimations of the independent variables.

$$Y = b_0 + (b_1 x_1) + (b_2 x_2) + (b_3 x_3) + (b_{11} x_1^2) + (b_{22} x_2^2) + (b_{33} x_3^2) + (b_{12} x_1 x_2) + (b_{23} x_2 x_3) + (b_{31} x_3 x_1) \quad (2)$$

A statistical program was utilized for regression examination of the data got and to gauge the coefficients of the regression equations. Examination of difference (ANOVA) was used for graphical examination of the data in order to acquire data of process elements with the

response. The nature of fit of polynomial model conditions was communicated by the coefficient of determination R^2 .

2.9. Determination of antioxidant activities

2.9.1. DPPH radical scavenging assay

The DPPH examine was resolved by the technique for Duan *et al.* (2006). 0.5 mL of the sample at various concentrations was blended with 0.5 mL of 0.16 mM DPPH in methanol and afterward incubated at 37 °C for 30 min in dark. The absorbance was measured at 517 nm. The rate of DPPH radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = \left(1 - \frac{\text{OD}_{\text{Treated}}}{\text{OD}_{\text{Control}}} \right) \times 100 \quad (3)$$

2.9.2. Reducing power

The reducing power was measured by the technique for Chou *et al.*, (2009). The sample at various concentrations (100-500 µg/mL) in ethanol (0.25 mL) was added to 0.25 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.25 mL of 1% potassium ferricyanide. At that point, the blend was brooded at 50 °C for 20 min. The 0.25 mL of 10% trichloroacetic acid was added to the blend to stop the reaction, and afterward the blend was centrifuged at 12,000 g for 10 min. The supernatant (0.4 mL) was mixed with 0.4 mL of ethanol and 80 µL of 0.1% ferric chloride solution and kept undisturbed for 10 min, and the absorbance was measured at 700 nm.

2.9.3. ABTS radical scavenging activity

ABTS radical scavenging activity of lutein was resolved by the technique for Re *et al.*, (1999) with a few changes. The radical ABTS^{••} was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate buffer (1:1) and kept for 16 h in dark at room temperature. After 16 h, ethanol was utilized to adjust the absorbance of the radical ABTS^{••} to 0.75 ± 0.07 at 734 nm. 1.9 mL of the radical ABTS^{••} was added to 100 µL of the sample at different concentrations. Butylated hydroxytoluene (BHT) is used as standard. The blend was remained at room temperature for 6 min, and afterward the absorbance was measured at 734 nm. Scavenging activity was calculated using the above equation (3).

2.9.4. Hydrogen peroxide radical scavenging activity

Hydrogen peroxide radical scavenging activity was resolved by the technique for Muller (2001). The reaction mixture comprised of 20 µL of 2 mM H₂O₂, 100 µL of 0.1 M phosphate buffer (pH 5.0), and 100 µL of sample, and after that incubated at 37 °C for 5 min. At that point, 30 µL of 1.25 mM ABTS and 30 µL peroxidase (1 unit/mL) were added to the reaction mixture and incubated at 37 °C for 10 min. The absorbance was measured at 405 nm. The rate of hydrogen peroxide radical rummaging movement was calculated using the above equation (3).

3. Results and Discussion

3.1. Characteristics of wastewater after biomass recovery

Microalgae play an important role in biological processes coupled with bioremediation of wastewaters due to its competence to assimilate organic compounds and nutrients. Fig. 2. elaborates the characteristics of wastewater after biomass recovery. Wastewater with

elevated concentrations of Phosphorus, Nitrogen, COD and TSS when discharged into the water bodies, leads to contamination of water bodies. To prevent this dilemma, our results show that microalgae treatment is economically viable process for treating the waste water effluents prior to dumping into the water bodies. The concentrations of Phosphorus, Nitrogen, COD, and TSS are reduced by about 97.93 %, 97.7 %, 91.08 % and 88.73 % respectively.

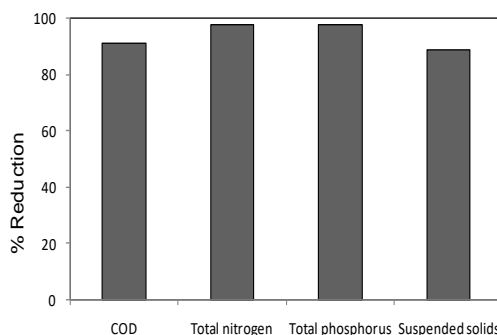


Figure 2. Characteristics of wastewater after biomass recovery

3.2. Initial trials for RSM optimization

Pre-RSM extraction trials for the variables incubation time, biomass concentration, and HCl % were studied in the following ranges: 15 min–45min (increments of 15 min), Biomass concentration 0.5– 3.0g (increments of 0.5), and 5–15 % HCl (increments of 5 %). The effect of HCl concentration on lutein yield was studied and results were elaborated on Fig.3A. The effect of incubation time (15-45mins) on lutein yield was studied and results were elaborated on Fig. 3B. At fixed 10% HCl concentration with 30mins incubation time resulted in a higher lutein yield. Accordingly then, the ranges were fixed for the upper and lower limits for the RSM design of experiment. The better fit between the varying incubation time (15-45min) and HCl concentration (5-15%) was studied and furnished in Fig.3C. Here, the 30min incubation time and 10% HCl concentration emerged as better conditions for the study. Fig 3D, elaborates the different conditions (HCl, Ultrasound, Ultrasound + HCl) adopted and their effectiveness for the lutein yield. Ultrasound with HCl extraction procedure showed a higher lutein yield than other two methods.

3.3. Spectral characterization of carotenoid lutein

Usually, to obtain pure carotenoid compounds practically a few phases of purification process are required. The spectrophotometric components of carotenoids are used to recognize and quantify the individual carotenoid lutein that has been purified. The spectrophotometric examination of the crude extract was analyzed from 300 to 600 nm. Because of extensive conjugated polyene chain, carotenoids show yellow, orange, and red colors (Landrum, 2010). They have retention maxima in the range of 420-480 nm. Thusly, carotenoids can show a unique molecular

shape, compound reactivity, and light fascinating properties.

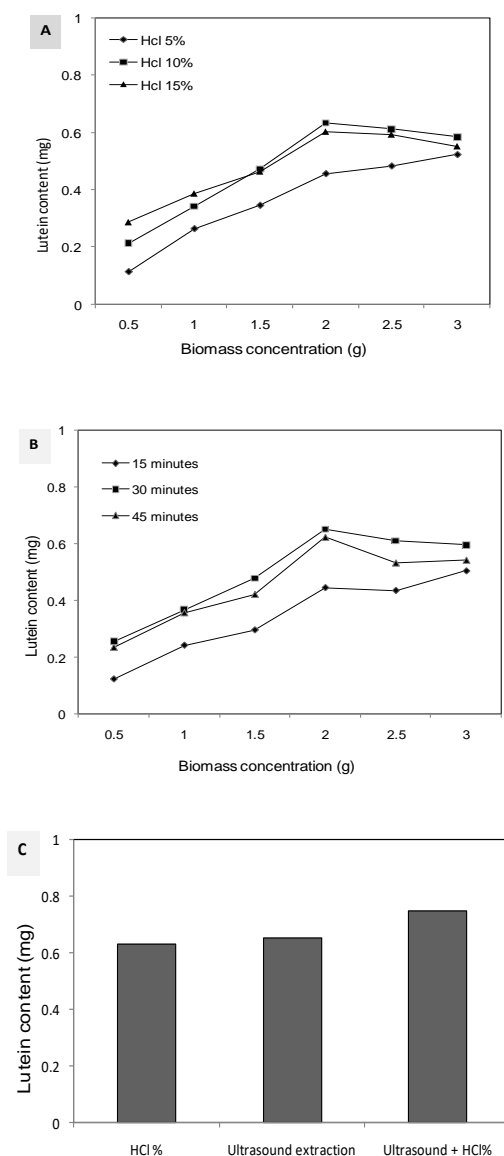


Figure 3. Initial trials for Lutein yield 3A) Effect of HCl%, 3B) Effect of ultrasound duration, 3C) Maximum lutein yield from different methods

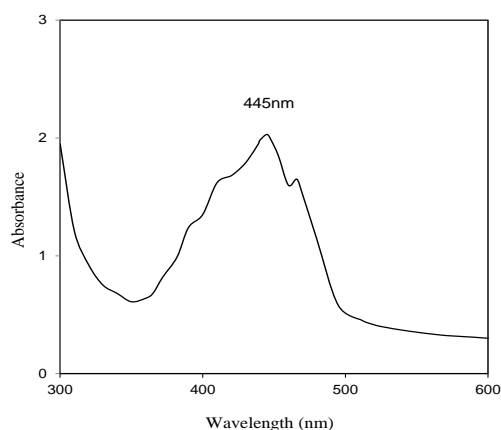


Figure 4. UV-visible spectrum of the extracted Lutein

3.4. HPLC analysis

The carotenoid profile was assessed by HPLC after a reference extraction procedure. As was found, absorbance disclosure at 445 nm allows the confirmation of the carotenoids down to the chromatogram stretches out with awesome linearity (Huck *et al.*, 2000).

Partition of lutein was performed on C18 turn column and an UV photodiode cluster pointer. Figure 5(A) standard peak (10 $\mu\text{g mL}^{-1}$); Fig. 5(B) plots the separation of lutein in the chromatogram at 4.52 min from *C. pyrenoidosa*.

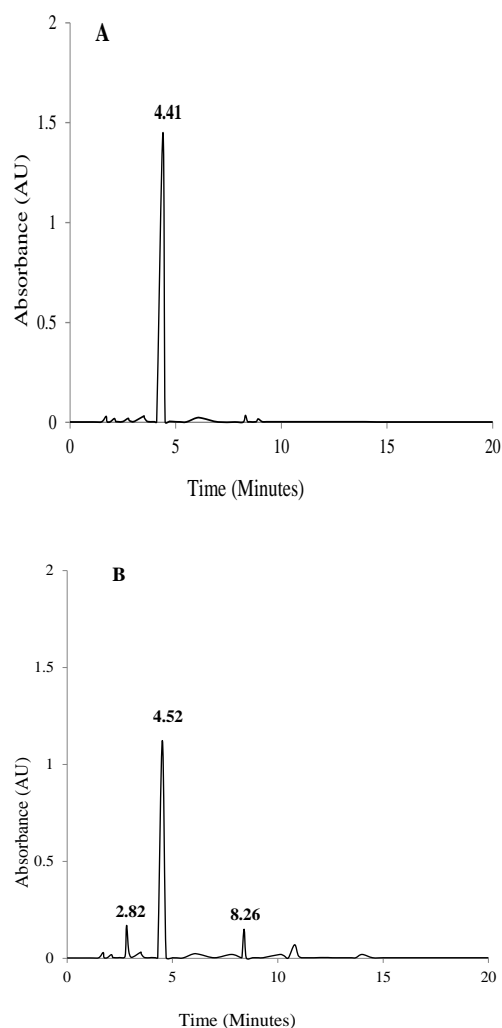


Figure 5. HPLC Chromatogram of the microalgal Lutein at 445 nm. 5A) Standard peak; 5B) microalgal lutein compound.

3.5. Micro algal cell morphology

The microalgal cell morphology was determined by scanning electron microscope. The pictures of *C. pyrenoidosa* treated by ultrasound with/without acid pretreatment are shown in Fig. 6. The microstructure of the microalgal cell by ultrasound (Fig. 6C) had more interspaces and openings than the microalgal cell by the acid treatment system (Fig. 6B). The disturbance in the microalgal cell walls by ultrasound extraction is showed up in Fig. 6C. This was the same as the results of (Zhao *et al.*, 2012; Li *et al.*, 2012)

in which ultrasound treated damaged tissues and cell walls. In addition, the physical unsettling influence of *C. pyrenoidosa* cells by ultrasound, together with acid pretreatment, was the most effective technique to destroy microalgal cell wall (Fig. 6D).

The cell walls were harmed by the usage of ultrasound and acid pretreatment, which brought about the more prominent penetration of solvent into the sample matrix, extended the contact surface region between the solid and liquid phase, and in this manner, the solute immediately diffused from the solid phase to the solvent. Henceforth, ultrasound treatment is significantly more gainful and quick for the extraction of the bioactive compound. Therefore, sonication is expected as a crucial part in separating the microalgal cell walls to upgrade the extraction yield (Chua *et al.*, 2009).

Table 2. ANOVA table for design analysis.

Source	Degree of freedom	Adj SS	Seq SS	Adj MS	F	P
Regression	9	4.09205	4.09205	0.45467	34.12	0
Linear	3	3.1561	3.1561	1.05203	78.95	0
Square	3	0.3982	0.3982	0.13273	9.96	0
Interaction	3	0.53775	0.53775	0.17925	13.45	0.001
Residual error	10	0.13325	0.13325	0.01332		
Lack of fit	5	0.0439	0.0439	0.00878	0.49	0.773
Pure error	5	0.08935	0.08935	0.01787		
Total	19	4.2253				

3.6.2. Main effect plot

The effect of elements on the reaction (lutein yield) could be examined from the effect plots. The main effect plot for the incubation time, biomass concentration, and HCl % is appeared in Fig. 2. The individual centrality of these components can be foreseen from this plot. In traditional optimization, one variable is changed at time keeping remaining parameters as steady. While RSM takes a gander at the simultaneous effect of all the included components and thus, the main effect plot obtained in this study depicts not only the effect of progress of one variable furthermore a merged effect of progress in the elements, which is just imparted by considering one calculate record. The plot proposes that the change in incubation concentration and HCl % impacts the lutein yield.

3.6.3. Reaction surface plots

The response surface contour plots are shown in Fig. 3. From these plots, it can be deciphered that the %lutein yield is higher at 10 % of HCl concentration and incubation time of 30mins. The yields of the lutein were 3.78 mg/g (Cha *et al.*, 2010) and 9.22 mg/g dry weight of *C. vulgaris* by ultrasound extraction, in which the dried *C. vulgaris* was used as an unrefined (crude) material. Freeze drying or lyophilization has been extensively used for drying microalgae in research labs; regardless, freeze drying is unreasonably expensive for use in a tremendous scale business recovery of microalgal products (Sevilla *et al.* 2010).

3.6.4. Global solution

3.6. Optimization studies

3.6.1. Experimental design analysis

The batch runs were conducted with experiments designed through CCD to visualize the effects of independent factors on responses and the results were evaluated. Approximating function obtained for lutein yield is given as

$$Y = 1.525 + 0.0137X_1 + 0.0463X_2 - 6.827 \times 10^{-4} X_3 - 6.749 \times 10^{-4} X_1^2 - 0.00303X_2^2 - 4.932 \times 10^{-6} X_3^2 + 0.0003X_1X_2 + 0.000155X_1X_3 + 0.000225X_2X_3 \quad (4)$$

ANOVA results of these quadratic models are presented in Table 2, indicating that these quadratic models can be used to navigate the design space.

Global solution for predicted reactions for the factors, for example, HCl concentration, incubation time and biomass concentration are fitted and cross checked with the diverse examinations of CCD plan with the parameters Incubation time = 41.34 min, HCl concentration = 15.63 % and Biomass concentration = 309.21 g/L which brings about a 3.5mg of lutein yield per gram of microalgae through the extraction technique.

3.7. Antioxidant activity

It is outstanding that the assessment of the antioxidant capacities on a chosen antioxidant requires more test frameworks (Yu *et al.*, 2002). The DPPH free radical is a steady free radical that has been generally utilized as an instrument for evaluating the free radical- scavenging activity of antioxidant (Nagai *et al.*, 2003). The ABTS is broadly utilized in food analysis since it is not subject to pH varieties and is useful to analyze both hydrophilic and lipophilic mixes (Zanfini *et al.*, 2009). Hydrogen peroxide itself is not extremely responsive, but rather it can once in a while be harmful to a cell, since it can bring the hydroxyl radicals to greater extent. In this manner the removal of H₂O₂ is important for antioxidant defense in cell or food systems (Subhashini *et al.*, 2011). The reducing capacity of a compound may fill in as a critical marker of its potential antioxidant activity (Duh, 1998). DPPH radical scavenging movement of microalgae is exhibited in Fig.7A. The lutein haunted the capacity to scavenge DPPH at different degrees; with the 250 µg concentration of *C. pyrenoidosa* (81.25%) was bring into being the most potent scavenger

Ferric reducing action of microalgae lutein is exhibited in Fig 7B.

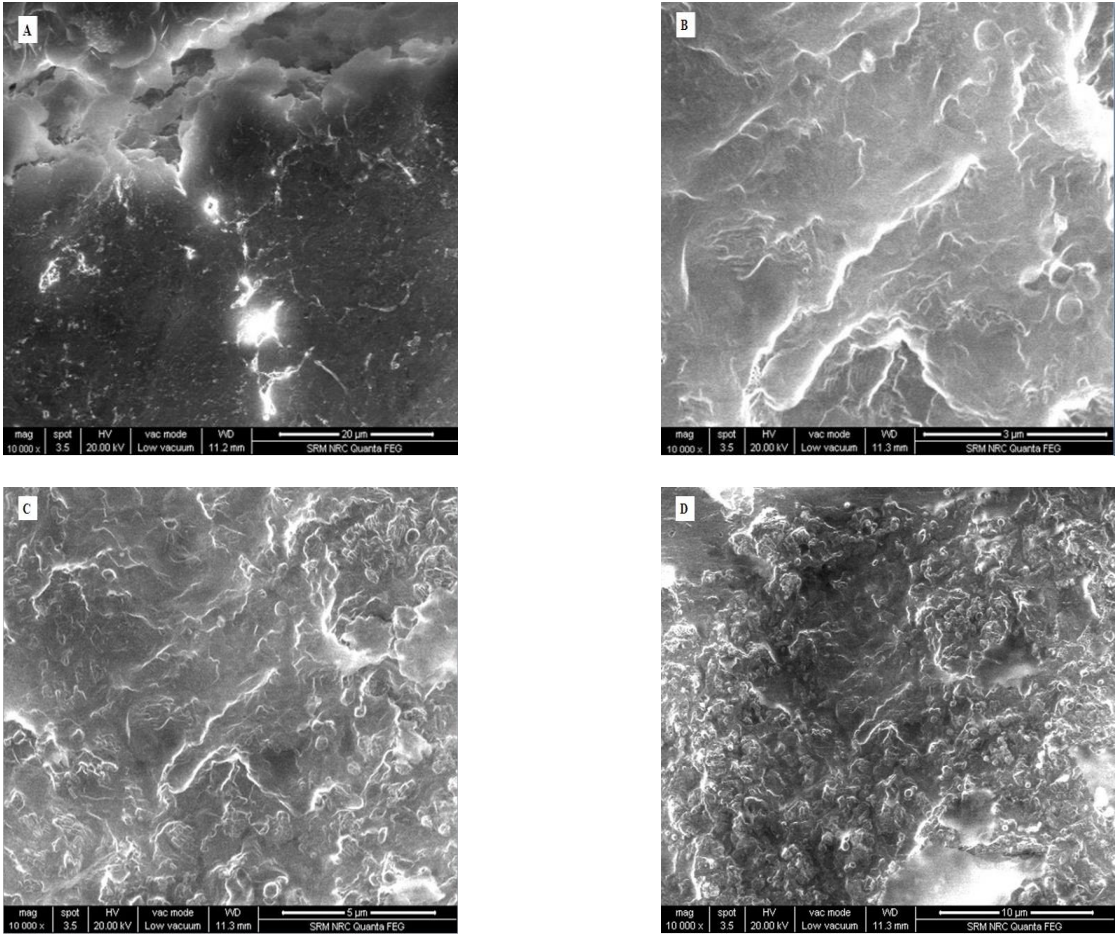


Figure 6. Scanning electron microscope images of *C. pyrenoidosa*. (A) Control; (B) Acid extraction; (C) ultrasound extraction; and (D) ultrasound extraction with acid pretreatment

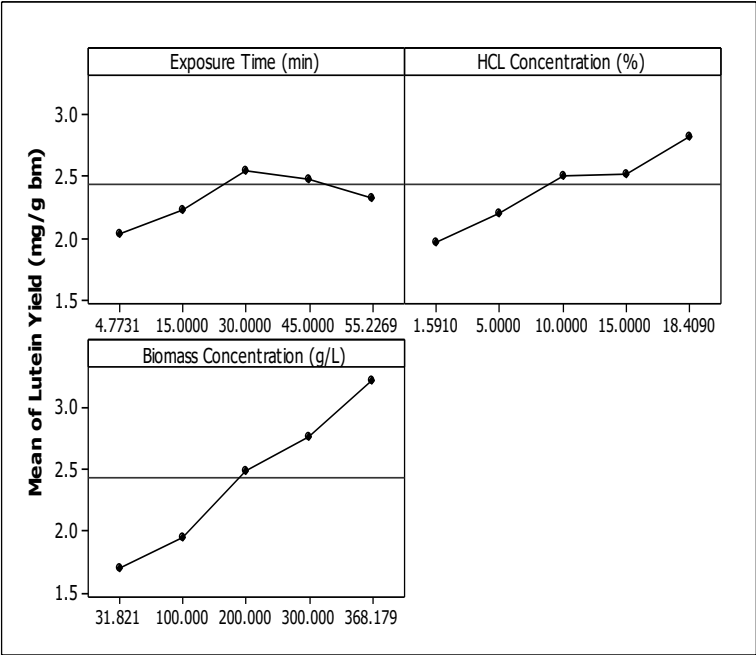


Figure 7. Main effect plot for incubation time, biomass concentration, and HCl %

The reducing power was found to be higher in 500 μg concentration of *C. pyrenoidosa* (0.783 mg/g ascorbic acid equivalent). Recently, the ethanolic extract of *Enhalus acoroides* exerted the potential antioxidant and ferric reducing activity (Sacristan *et al.*, 2013) which is higher or similar to our study. ABTS radical scavenging activity of microalgae lutein is presented in Fig. 7C. ABTS radical scavenging activity was found to be higher at 250 μg concentration which exerted 64.53 % of antioxidant activity than other concentrations. Recently, the microalgae's *Pterocladia capillacea* and *Osmundaria obtusiloba* also exerted more or less similar radical scavenging activity (Gao *et al.*, 2014).

4. Conclusion

Ultrasound assisted extraction was applied for the lutein yield from *C. pyrenoidosa*. The response surface technique

was chosen to obtain the ideal conditions of ultrasound extraction with/without acid pretreatment. The ultrasound extraction with acid pretreatment brought about the most remarkable lutein yield, trailed by ultrasound and acid extraction all together. In correlation with the acid extraction, ultrasound extraction is a more proficient and fast technique to separate the lutein from *C. pyrenoidosa*, because of the strong disruption of the cell wall under ultrasonic acoustic cavitation. Likewise, they had a promising anti-oxidant action which shows that this microalga can be additionally taken up for these free radical rummaging exercises. Along these lines, it is recommended that ultrasound extraction with acid pretreatment is the effective technique for the extraction of lutein.

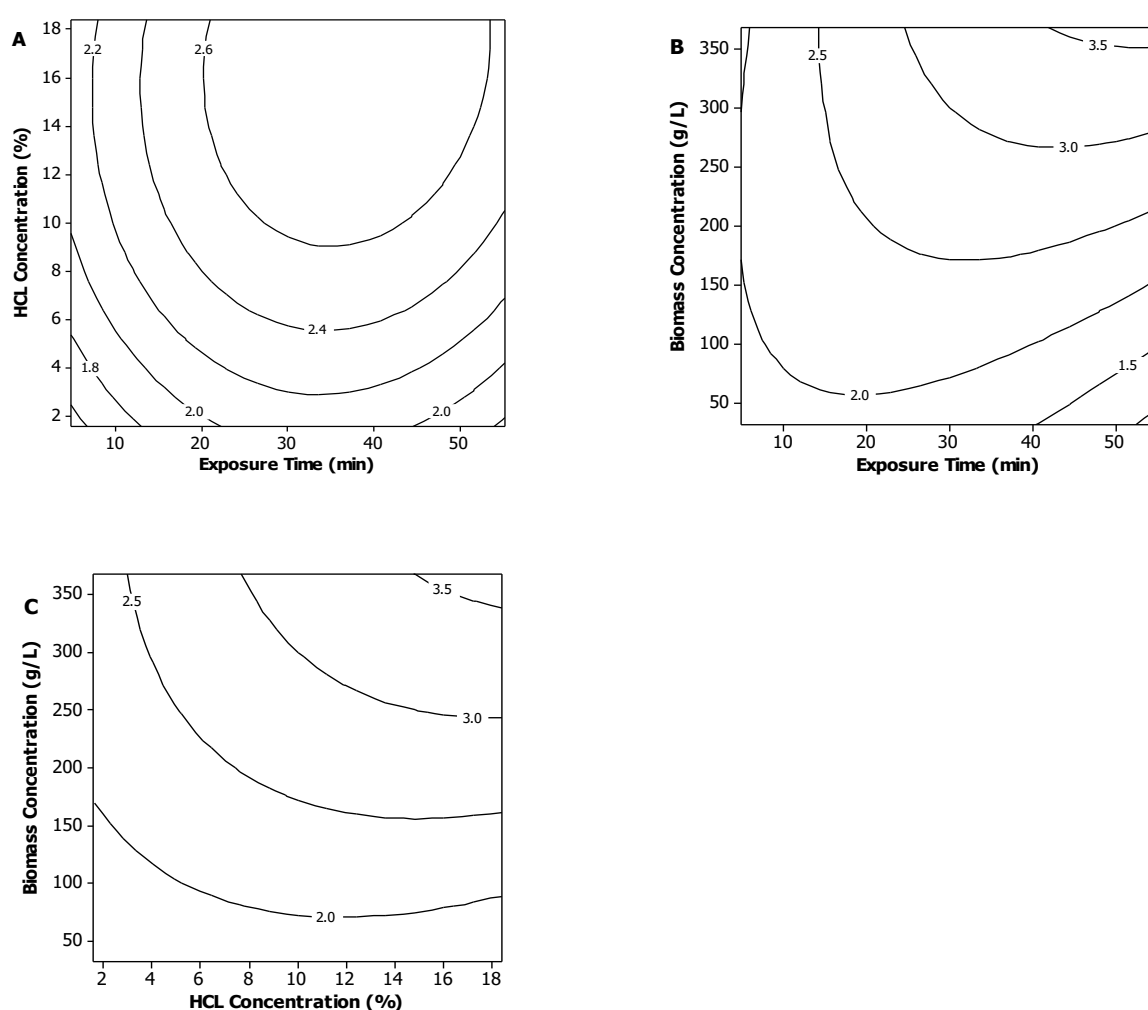


Figure 8. Multiple response surface optimisation plots for the yield of lutein from *C. pyrenoidosa* by ultrasound extraction as a function of (A) HCL concentration and incubation time, (B) biomass concentration and incubation time, and (C) biomass concentration and HCL concentration.

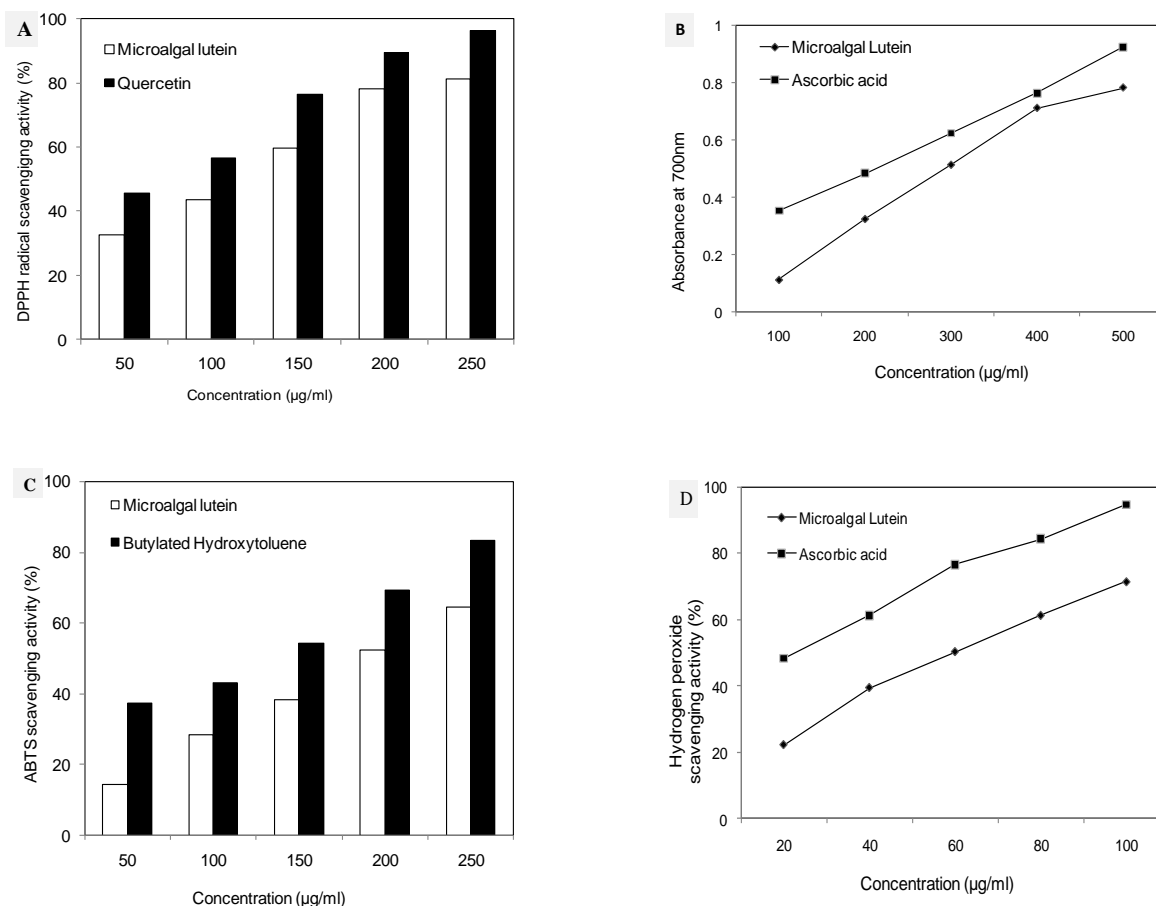


Figure 9. Antioxidant activities of microalgal lutein at different concentrations by ultrasound extraction: (A), DPPH; (B), Reducing power; (C), ABTS; (D), Hydrogen peroxide assay

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Conflict of interest

Authors declare we have no conflict of interest

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