Perspectives of Lipid Extraction By Biomass Stages of Microalgae *Aphanothece Halophytica* Developed in Business Scale

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Abstract

Worldwide, people are accepting a sustainable, renewable and economical source for biofuel production without affecting the environmental concerns. Microalgae can overcome problems with reasonable solutions along with higher lipid content, carbon neutrality, biomass productivity and utilization of feed supplements. Picking a distinctive strain is vital and the value depends on its micro, macro nutrients and its photosynthesis efficiency. Extracting oil from the different phases of microalgal biomass is a tedious process. Presently, halophilic *Aphanothece halophytica* was mass cultivated in raceway ponds using organic and inorganic nutrients. Microalgal biomass was harvested using novel organic flocculant and oil extraction was done through wet and dry basis based on solvent polarity. The process parameters were optimized for maximum lipid productivity using RSM and the empirical model was significantly analysed by analysis of variance. In wet basis, maximum lipid yield was 29.3%, where the reaction temperature, reaction time, biomass-to-solvent ratio and mixing intensity are 68ºC, 190 min, 9:1 and 300 rpm respectively. In dry basis, lipid yield 27.5% was achieved using 12:1 biomass-to-solvent ratio and mixing intensity was 300 rpm for 190 min at 68ºC. Then, the lipid was characterized by GCMS to identify the fatty acid composition to identify the right combination of fatty acid profile for further studies.

1. Introduction

Due to increase in fuel cost and continuous depletion of crude oil associated with environmental issues there is an urge to search an alternative which is clean, renewable and sustainable. Recent studies established positive results in microalgal fuel with environment concern and fossil fuels dependence. Microalgae are promising source for high lipid content which has fast growing rate and can cultivate in waste surface. Moreover, a microalga does not compete with food feedstocks and makes it a stimulating substitute for popular food and non-food crops [1, 2].

Energy recovery from microalgae is a necessary footstep to enhance and improve the sustainability and economic competitiveness. Releasing the lipid from the complex structure of microalgal cell wall is a major tricky in the extraction process [3, 4]. Usually, microalgal biomass was subjected to taking out the lipids by mechanical pressing and organic solvents. In mechanical pressing, the yield of oil depends on the extraction technique employed such as screw / piston press, extruder and expander, pulverization using mortar, etc [5, 6]. The method of organic solvent extraction employs ‘like dissolving like’ concept of basic chemistry to extract lipid from microalgae using solvents [7].

In the cell membrane of microalgae, neutral lipids are found as a complex with polar lipids and are strongly linked with the proteins through hydrogen bonds. Ideal solvent must be highly specific and volatile in order to ensure low energy distillation for extracting lipid from the solvent [8]. The non-polar solvents used for lipid extraction from microalgae biomass include hexane, benzene, toluene, diethylether, chloroform, chloroethane and dichloromethane. Similarly, the polar solvents used are methanol, ethanol, acetone, ethyl acetate and 2-propanol. The non-polar solvents disrupt the hydrophobic interactions between non-polar and neutral lipids, whereas the polar solvent disrupts the polar lipids. Addition of both

polar and non-polar organic solvent ensures, complete extraction of all neutral lipid (free standing globules and membrane associated complexes). The combinations of polar and non-polar solvent generally used are chloroform/methanol, n-hexane/ethanol, n-hexane/2-propanol, chloroethane/methanol, dichloroethane/ethanol and acetone/dichloromethane [9, 10]. However, chloroform/ methanol (1:2 v/v) is the mostly used organic solvent because of the reduced extraction time and high yield.

The remaining water in the algal cells acts as a ternary component, permitting complete extraction of both neutral and polar lipids. In this method complete drying of biomass is not needed, since separation happens by biphasic partitioning. The lower phase (chloroform and some methanol) contains lipid (neutral and polar), while the upper aqueous phase (water, methanol) contains non lipid components (proteins and carbohydrates) [10].

One hypothesis speculates that the presence of residual moisture in microalgal biomass unfavourably affects the lipid extraction efficiency. In such school of thought, the water forms a barrier and inhibits effective lipid mass transfer from cells to the organic solvent. So drying of microalgal concentrate is recommended prior to lipid extraction [9]. Another hypothesis postulates that the presence of residual moisture content in microalgal biomass will enhance lipid extraction efficiency. The principle is that the water swells the cells and allows better solvent access to the lipids and states that drying of microalgae before lipid extraction is unnecessary and may hinder the lipid extraction [11].

Soxhlet or hexane solvent extraction method can be used either alone or in combination with oil press method. Oil extraction in batch mode needs large amount of organic solvent, whereas continuous mode replenishes the biomass with organic solvent and reduces the solvent consumption [12, 9]. This method is quite expensive and has to be handled carefully as it involves chemicals. Benzene is a carcinogen and can lead to explosion hazard also. Hexane is less efficient than chloroform which is less toxic and has low affinity towards non lipid contaminants [13]. Bligh and Dyer's method is used for both dry and wet mode of oil extraction [14] where the ratio of chloroform/methanol is maintained around 2:1. After mixing the solvent and the biomass, it is homogenized with another same quantity of solvent. The method of centrifugation separates the biphase layer (lipid in chloroform and methanol in water) in the process and the lipid is separated finally by fractional distillation [15, 16].

There is an emerging green technology for oil extraction where super critical carbondioxide ($SCCO_2$) is used as the primary solvent at a critical pressure of 7.4 MPa and at lower critical temperature of 31.3ºC [17, 18]. The factors affecting this process include the pressure, the temperature and the fluid flow rate. $SCCO_2$ has high solvatic power and is less toxic, but the high infrastructure requirements and the operating cost are the main challenges in this process [19, 20,]. A contrasting result has been reported in a comparative study between $SCCO_2$ and Bligh and Dyer method for extracting heterotrophically cultured microalgae *C. cohnii* [21, 22]. The lipid yield obtained from Bligh and Dyer procedure is nearly double compared to this method. It is noted that the microalgae strains and culture conditions play a crucial role in the determination of appropriate lipid extraction method. The microalgal residues are rich in

carbohydrates, proteins and pigment [23, 24, 25, 26, 27]. They can be further processed into biomethane, bioethanol, biobutanol, etc. [28, 29, 30, 31, 32, 33]. It is also used to produce valuable products like docosahexaenoic acid (DHA), carotenoids, drugs, food and feed additives [34, 35, 36].

In this work, microalgae *Aphanothece halophytica* was isolated from saltpan area and be cultivated in Jaworski's medium. Then, the culture was mass propagated using organic and inorganic combo nutrients in open raceway ponds. The lipid was extracted by two ways 1) from wet biomass and 2) dry biomass. According to the polarity, solvents were selected for both methods. The extraction process parameters such as extraction time, temperature, biomass-to-solvent ratio and mixing intensity were optimized by response surface methodology (RSM). The lipid was quantified and proposed a better practice for commercialization. Finally, lipid was characterized by gas chromatography to obtain the fatty acid signature for further processes.

2. Materials And Methods

The working methodology of the lipid extraction enhancement process from the microalgae isolation is charted in the Fig. 1.

2.1. Microalgae culture Protocol

Water samples were collected from saltpan (8.1089^0N, 77.4667^0E), Kanyakumari District and the physiochemical characteristics were analyzed. The samples were enriched by using standard marine Jaworski's medium followed by serial dilution. Then purified by quadrant streaking using solid medium. Then the inoculated cultures were incubated at 25ºC with an illumination of 2500 lux for 12:12 h light/dark at pH 8.2 for about one month in the laboratory. Morphological depiction was done using a microscope and Nile Red staining was employed for visualizing the presence of lipid which was published in Elsevier [37].

2.2. Oil extraction procedure

Extraction of lipid was done by Bligh and Dyer method from the dried biomass. Powdered biomass of about 1 g was mixed with 30 ml of chloroform/methanol (2:1) and was gently agitated in an orbital shaker at 100 rpm for 20 min and then centrifuged at 2000 rpm for 10 min to separate the oil extract and the microalgal pellet. The resultant oil extract was allowed to evaporate at 40ºC and then kept in hot air oven at 70ºC for 2 h [16]. Then the lipid was quantified and the lipid productivity was estimated [38].

2.3. Large scale propagation methodology

2.3.1. Preparation of Inoculum

Ten percentage of the log phase microalgal culture was inoculated in the 1 l flask containing the media, followed by 10 l flask, 20 l flask and 200 l glass tank with the same conditions [37]. The seed culture for cultivation in open raceway ponds was separately grown in 500 l capacity round cement tanks. Initially

the tank was thoroughly rinsed with 5% sodium hypochlorite in tap water and kept overnight in closed condition. The tank was again rinsed with tap water and exposed to sunlight for 12 h. 175 ml of seaweed extract + NPK solution along with 350 l of filtered sea water was loaded onto the tank, where 35 l of log phase inoculum was added, which was grown in a glass tank. The pH and the salinity were maintained at 8.2 and 60 ppt respectively.

2.3.2. Raceway Pond Cultivation

For mass propagation, the microalgae were cultivated in open raceway ponds of size 7.6×1.2×0.4 m having the total volume of 3500 l capacity. The microalgal growth medium was prepared with the optimized condition of seaweed extract + NPK in 3300 l of filtered sea water. 10% of starter inoculum culture from the round cement tank was added to initiate growth in the raceway pond. The medium was continuously agitated using a paddle wheel at 36 rpm, to prevent algal culture sedimentation and ensure uniform acquaintance of all microalgae to light and nutrients. Gentle agitation is needed to avoid thermal stratification and to enhance CO_2 distribution. Culture purity was ensured through regular microscopic examination.

2.3.3. Mode of cultivation

The mode of cultivation is semi-continuous and provides partial periodic harvesting. Initially 25% of the microalgal culture was collected on the peak day and then on daily basis. The pH was maintained at 8.2 and the volume of culture harvested was replenished with appropriate amount of fresh nutrient to maintain the optimum culture conditions.

2.4. Biomass Harvesting

The harvesting of microalgal cells was done by bio flocculation process using organic flocculant, commercial Neem plus. Experimental investigation was done to optimize the required flocculant dose by varying the concentration as 0.001, 0.002, 0.003, 0.004, 0.005 ml/l and the optimized dose was adopted for biomass harvesting.

2.5. Lipid Extraction Procedure

Oil extraction was done on wet basis as well as on dry basis. In wet method, the harvested biomass was dewatered with cheese cloth and kept in an autoclave for 20 min at 121°C and 15 psi pressure whereas in dry mode of extraction, after pressing the harvested biomass with cheese cloth, it was spread on polythene sheets and sun dried followed by microwave oven drying at 50ºC. The dried microalgal biomass was then powdered by using a pulveriser. The moisture content of the microalgal biomass was determined for every process.

Selection of appropriate solvent is important for efficient and economical extraction of algal oil. Based on the literature and confirmation test chloroform/methanol (2:1), a non-polar/ polar solvent mixture was selected. The properties of chloroform and methanol are indicated in Table 3.1.

Table 1
Properties of chloroform and methanol solvent

Property	Chloroform	Methanol
Chemical formula	$CHCl_3$	CH_3OH
Nature	Non polar solvent	Polar solvent
Molar mass	119.37 g/mol	32.04 g/mol
Appearance	Colorless	Colorless
Odor	Ethereal odor	Alcoholic odor
Boiling point	61ºC	65ºC
Melting point	-63 ºC	-98ºC
Density	1.492 g/cm^3	0.791 g/cm^3
Auto ignition point	455ºC	982ºC

2.5.1. Extraction Setup

The experimental setup comprises of a three neck round bottomed flask and a hot plate temperature controlled magnetic stirrer (Fig. 2). The large neck of the flask was allied to the reflux condenser and the other two ports are meant for a thermometer and sampling. Designed quantity of solvent was added with 25 g of biomass and heated at various designed temperature for different duration at various intensity. After the process, the blend was permitted to cool and filtered to remove the biomass. The filtrate was distilled using rotary evaporator to separate the lipid from the solvent and it was quantified after removing the moisture by heating at 105°C for 1 h. Then, the acid value was found through the titration process to confirm the steps involved in the biodiesel conversion process.

2.5.2. Optimization of microalgal oil extraction

A three level four factor Box-Behnken Design (BBD) was employed in the optimization process, with 29 sets of experiment, contains 5 central points and 24 axial points. The extraction process parameters such as biomass-to-solvent ratio, mixing intensity, extraction temperature and time are to be enhancing the exploit of oil yield. The range and level of the factors involved in oil extraction for wet and dry biomass is shown in Table 2.

Table 2. The ranges and levels of the lipid extraction process parameters using wet and dry biomass

Biomass phases	Process parameters	Unit	Range and level		
			Low (-1)	Medium (0)	High (+1)
Wet biomass	Biomass-to-solvent ratio	(v/w)	4:1	8:1	12:1
	Extraction time	min	60	180	300
	Extraction temperature	(ºC)	55	65	75
	Mixing intensity	rpm	200	450	700
Dry biomass	Biomass-to-solvent ratio	(v/w)	6:1	10:1	14:1
	Extraction time	min	60	210	360
	Extraction temperature	(ºC)	55	65	75
	Mixing intensity	rpm	300	600	900

2.6. Lipid characterization by GC-MS

Perkin Elmer Clarus 500 spectrometry was used to find the fatty acid profile of the microalgal lipid with mass detector (Turbo mass gold- Perkin Elmer) and column ELITE 1-100% Dimethyl poly siloxane, (30×0.25mm×1μmdf) under nitrogen atmosphere (1ml/min). Primarily, the temperature was retained at 110ºC for 2 min and then it was increased at the rate of 5º C/min till 208ºC. 2 μl of the sample was given to the spectrometer which is fitted with the mass spectrometer. The profile of the fatty acid was identified through the percentage of peak areas with their retention time.

3. Results And Discussion

3.1. Mass propagation analysis

The isolated *Aphanothece halophytica* cultivated in the lab scale was then cultured in outdoor open raceway ponds (Fig. 3). This process has better possibility to achieve higher biomass production [39]. The best medium recognized after methodical study for exploiting the oil productivity was seaweed extract + NPK with 0.5 ml/l, 0.05 g/l urea, 0.5 g/l SSP and 0.5 g/l MOP [37]. In general, open pond system needs specific environment for a selected species to avoid contamination and pollution from other microbial species [40, 41, 42]. Monoculture cultivation of *Aphanothece halophytica* was possible since it thrives very well in high salinity conditions. Similarly such successful large scale monoculture cultivation of *Acutodesmus dimorphus* was reported in extreme halophilic condition as well [43]. It is also reported that heterotrophic cultivation of microalgae results in higher biomass and lipid content [44].

The biomass was harvested on the log max day (20th day) using flocculation method by organic commercial neem plus solution which is a low energy consuming method to harvest microalgae. Preliminary experiments were conducted to optimize the required dose of organic flocculant. The observed outcomes are given in Table 3, which reveals that, the concentration of 0.002 ml/l of flocculate

was enough for harvesting the maximum microalgal biomass within 180 sec. being an organic flocculant, it will avoid the usage of costly toxic chemicals for microalgal harvesting. Similar methodology has been used in the used in many microalgal biomasses harvesting using Chitosan [45, 46]. For example, microalgae *Microcytis aeruginosa* was effectively harvested using chitosan, with an efficiency of 99.2% [47] It was reported that harvesting of *Chlorella vulgaris* using 120mg/l of chitosan gave an efficiency of 92 ± 0.4% within 180 s [48]. Moreover, organically flocculated medium can be reused effectively without affecting microalgal growth. This will reduce the overall production cost by avoiding the process for water treatment and purification [46, 49]. In the present work, the higher yield 300 g of dried biomass per day was attained after cultivation of *Aphanothece halophytica* in 6 raceway ponds.

Table 3
Effectiveness of biomass harvesting using organic flocculant, neem plus

Neem plus (ml/l)	Harvested biomass (%)	Time of harvesting (s)
0.001	94	250
0.002	99.4	180
0.003	99.1	180
0.004	99	179
0.005	98	179

3.2. Optimization of lipid extraction parameters

3.2.1. Lipid Extraction from Wet Biomass

The wet biomass yield obtained after harvesting is shown in Fig. 4. The major parameters influencing the extraction of lipid from wet biomass are temperature, time, biomass-to-solvent ratio and mixing intensity. The range and level of each parameter were confirmed by pilot experiments in corroboration with literature. The designed experiments and the combination of process parameter along with their corresponding lipid yield are tabulated in Table 4.

Table 4
The Box-Behnken designed process parameters (BBD) for optimizing the extraction process on wet basis and their corresponding lipid yield

No. of run	A:Extraction Temperature (°C)	B:Extraction time (min)	C: biomass-to-solvent ratio (v/w)	D:Mixing intensity (rpm)	Lipid yield (%)
1	65	60	8	700	17.4
2	65	180	4	700	8
3	55	300	8	450	26.4
4	65	180	8	450	28.8
5	65	180	8	450	28.5
6	65	60	4	450	0.7
7	65	300	12	450	28.9
8	65	180	12	700	25.8
9	75	60	8	450	17.5
10	55	60	8	450	10.5
11	65	180	12	200	26.9
12	55	180	8	700	20.2
13	65	300	8	700	27.5
14	65	180	4	200	7.22
15	75	180	8	200	21.8
16	75	300	8	450	22.5
17	55	180	4	450	5.1
18	65	60	12	450	19.7
19	65	60	8	200	17.3
20	75	180	4	450	4.4
21	65	180	8	450	28.7
22	65	180	8	450	28.9
23	75	180	8	700	22.9
24	65	180	8	450	28.7
25	55	180	12	450	21.7

No. of run	A:Extraction Temperature (°C)	B:Extraction time (min)	C: biomass-to-solvent ratio (v/w)	D:Mixing intensity (rpm)	Lipid yield (%)
26	65	300	4	450	11.6
27	55	180	8	200	21.4
28	65	300	8	200	28
29	75	180	12	450	25.3

The developed empirical model was verified for its significance and aptness by analysis of variance (ANOVA). The model p-value was less than 0.0001 indicating statistical significance. The analysis of variance results is tabulated in Table 5. The lack of fit was satisfactorily low, which is 0.1359 and so the model is fitted to the investigational data.

Table 5
Analysis of variance (ANOVA) results of the model to optimize the oil extraction process on wet biomass basis

Source of variables	Sum of squares	Df	Mean square	F-value	P-value Pro>F
Model	2080.01	14	148.57	2616.37	<0.0001 significant
A-Extraction Temperature	6.9	1	6.9	121.52	< 0.0001
B-Extraction time	319.3	1	319.3	5622.91	< 0.0001
C- biomass-to-solvent ratio	1033.79	1	1033.79	18205.14	< 0.0001
D-Mixing intensity	0.056	1	0.056	0.99	0.3374
AB	29.7	1	29.7	523.06	< 0.0001
AC	4.62	1	4.62	81.4	< 0.0001
AD	1.32	1	1.32	23.29	0.0003
BC	0.64	1	0.64	11.27	0.0047
BD	0.09	1	0.09	1.58	0.2286
CD	0.88	1	0.88	15.56	0.0015
A^2	174.16	1	174.16	3066.97	< 0.0001
B^2	110.73	1	110.73	1949.94	< 0.0001
C^2	581.3	1	581.3	10236.81	< 0.0001
D^2	28.38	1	28.38	499.75	< 0.0001
Residual	0.79	14	0.057	-	-
Lack of fit	0.71	10	0.071	3.21	0.1359 not significant
Pure Error	0.088	4	0.022	-	-
Cor Total	2080.81	28	-	-	-

The R^2 value, 0.9996 indicates good pact between the foreseen and the actual yield (Fig. 5). The Pred R-Square value of 0.9980 was in reasonable agreement with the Adj R-Square value of 0.9992. In this model, the ratio is 168.512 which indicates the adequacy of the signal to be used in the design space.

In addition to this, the normal probability plot of the residuals shows that the errors are spread in normal to a straight line insignificantly (Fig. 6). The Fig. 7, the residuals versus predicted response displays that

the spread is even across all points approving the competence of the model over its range [50].

The percentage of oil yield on wet basis predicted by the model in terms of coded factors is given in equation 1.

$$Yield = 28.72 + 0.76A + 5.16B + 9.28C - 0.068D - 2.72AB + 1.07AC + 0.57AD - 0.40BC - 0.15BD - 0.47CD - 5.18A^2 - 4.13B^2 - 9.47C^2 - 2.09D^2 \quad (1)$$

Extraction of lipid from wet microalgal biomass diminishes the energy requirement for the drying process [51, 52]. The perturbation plot (Fig. 8) depicts the effects of an extraction parameter on the lipid yield in the designed space while keeping the other process parameters constant at their midpoint. The steep positive slope (C-C) was observed for biomass-to-solvent ratio towards oil yield up to + 0.5 reference indicates that as the solvent quantity increases, the oil yield increases. However, the response gets saturated around 8:1 biomass-to-solvent ratio and further addition of solvent was redundant. The plot for the reaction time shows that as the reaction time rises there was increase in yield and get saturated around 300 min. The reaction temperature was optimum near the boiling point of methanol (65ºC) and deviation from this temperature shows a negative effect on biodiesel yield. The mixing intensity was to be kept at optimum for enhancing the reaction to maximize the yield. The influence of this parameter in the designed range was very minimum. Some researchers have reported that the negative influence of moisture on oil yield, which can be negated with pre-treatments by making the intracellular lipids more readily available for extraction by solvent [53, 54].

3.2.1.1. Influence of solvent biomass ratio and extraction time (wet basis)

The solvent, Chloroform/methanol for oil extraction is very effective to both neutral and polar lipids when compared with other solvents [55, 56]. For example, the non-polar solvent, hexane (4:1) dissolves only the non-polar lipids in the microalgae with an oil yield of 9.4% from olive foot cake [57]. Solvent mixture of toluene, n-hexane, ethanol and methanol was used to extract oil from sewage sludge, where the higher yield was obtained for the solvent biomass ratio of 5:1 [58]. Fig. 9 shows the 3D surface plot and corresponding contour plot for oil yield with respect to solvent ratio and time with a minimum of 7:1 solvent ratio was needed for higher yield when allowed to react for more than 150 min. The optimized empirical model predicts a maximum yield of 28.9% at 8:1 solvent biomass ratio. It was renowned that as the reaction time increases the oil yield increases and get saturated as time reaches maximum level in the designed domain. The optimized time considering the correlation effects among the process parameters was 190 min.

3.2.1.2. Influence of extraction temperature and mixing intensity (wet basis)

Solvent based extraction allows complete extraction of oil when carried out closer to the boiling point of the solvent [59, 60]. The influence of temperature on oil extraction from *A. halophytica* wet biomass was

investigated between 55ºC and 75ºC. The 3D surface plot and the matching contour plot show that the outcome of reaction temperature and stirring intensity on the lipid yield from wet biomass (Fig. 10). It was well-known that the oil yield was optimum around the boiling point of methanol (65ºC), where the effectiveness of the solvent was maximum. At optimum temperatures the evaporation rate and the condensation rate (reflux) balance perfectly for maximizing the oil yield. Temperature higher than the optimum leads to loss of solvents by evaporation. The solvents after extraction are recovered by simple distillation process.

Proper stirring was important to mix the biomass with the solvent for effective chemical reaction to occur and to enhance the oil extraction. Fig. 10 shows that the oil yield increases as the stirring speed increases and was appropriate around 450 rpm. However, the optimum stirring speed for maximum oil yield by considering the interactive effects of other process parameter was 300 rpm.

The optimum process parameters of oil extraction were statistically derived from the developed empirical model to make the most of the oil yield in a short time. The finest value of the predicted process parameters is listed in Table 6.

Table 6
The predicted optimized process parameters value for maximizing the oil extraction in short span (wet basis)

Reaction temperature (°C)	Reaction time (min)	Solvent biomass ratio (v/w)	Mixing intensity (rpm)	Oil yield (%)
68	190	9:1	300	29.3

3.2.2. Lipid Extraction from Dried Biomass

The obtained dried biomass obtained is shown in Fig. 11. The parameters influencing the extraction of lipid from dry biomass are the same as that for the extraction on wet basis. The designed process parameter combinations and their corresponding lipid yield are given in Table 7.

Table 7
The Box-Behnken design for optimizing the extraction process parameters on dry basis and their corresponding lipid yield

No. of run	A:Extraction Temperature (˚C)	B:Extraction time (min)	C:solvent biomass ratio (v/w)	D:Mixing intensity (rpm)	Lipid yield (%)
1	55	60	10	600	7
2	65	360	6	600	17.9
3	65	360	10	900	24.9
4	65	60	6	600	5
5	55	360	10	600	21.7
6	75	210	6	600	10.8
7	65	210	10	600	27.1
8	65	60	10	300	12.2
9	55	210	14	600	16.8
10	65	210	10	600	27.2
11	75	360	10	600	24.4
12	65	210	10	600	27.4
13	65	60	10	900	11.4
14	65	60	14	600	15.1
15	65	210	14	300	22.4
16	55	210	10	300	14.1
17	75	210	14	600	24.2
18	65	360	10	300	24.7
19	65	210	6	900	11.5
20	65	210	6	300	12
21	75	210	10	300	20
22	75	210	10	900	17.6
23	65	360	14	600	27.4
24	75	60	10	600	12.5
25	65	210	10	600	27.3

No. of run	A:Extraction Temperature (˚C)	B:Extraction time (min)	C:solvent biomass ratio (v/w)	D:Mixing intensity (rpm)	Lipid yield (%)
26	65	210	14	900	22.1
27	65	210	10	600	27.2
28	55	210	6	600	9.7
29	55	210	10	900	15.4

The developed empirical model from the experiments on dry basis was verified for their significance and fitness by analysis of variance (ANOVA) and results were tabulated in Table 8. The model p-value was less than 0.0001 indicating statistical significance. The lack of fit was low enough to ignore the value of 0.0522 that shows the model was perfectly fitted with the investigational data.

Table 8
Analysis of variance (ANOVA) results of the model to optimize the lipid extraction process dry biomass basis

Source of variables	Sum of squares	Df	Mean square	F-value	P-value Pro>F
Model	1362.7	14	97.34	1686.86	< 0.0001
A-Extraction Temperature	51.25	1	51.25	888.24	< 0.0001 significant
B-Extraction time	505.7	1	505.7	8763.95	< 0.0001
C-solvent biomass ratio	312.12	1	312.12	5409.14	< 0.0001
D-Mixing intensity	0.52	1	0.52	9.03	0.0095
AB	1.96	1	1.96	33.97	< 0.0001
AC	9.92	1	9.92	171.96	< 0.0001
AD	3.42	1	3.42	59.31	< 0.0001
BC	0.063	1	0.063	1.08	0.3156
BD	0.25	1	0.25	4.33	0.0562
CD	1.00E-02	1	1.00E-02	0.17	0.6835
A^2	237.36	1	237.36	4113.46	< 0.0001
B^2	148.62	1	148.62	2575.62	< 0.0001
C^2	229.57	1	229.57	3978.58	< 0.0001
D^2	119.19	1	119.19	2065.64	< 0.0001
Residual	0.81	14	0.058	-	-
Lack of fit	0.76	10	0.076	5.81	0.0522 not significant
Pure Error	0.052	4	0.013	-	-
Cor Total	1363.51	28	-	-	-

The R^2 value of the model was 0.9994 indicates better pact between the predicted and the actual yield (Fig. 12). Further, the normal probability plot of the residuals shows that the errors were dispersed in normal by a straight line insignificantly (Fig. 13). The Fig. 14 shows the spread of residuals versus predicted response and it is uniform across all levels approving the suitability of the model over its range [45].

The percentage of oil yield on dry basis predicted by the model in terms of coded factors is given in Equation 2.

$$Yield = 27.24 + 2.07A + 6.49\,B + 5.10C - 0.21D - 0.70AB + 1.57AC - 0.92AD - 0.12BC + 0.25BD + 0.050CD - 6.05A^2 - 4.79B^2 - 5.95C^2 - 4.29D^2 \quad (2)$$

It was found that the efficiency of lipid extraction decreases with moisture content in biomass, hence the oil extraction analysis was done on dry basis also [61]. Most influential process parameters were studied by design of experiments and the corresponding experimental results were analyzed using ANOVA to draw conclusion from the model. The perturbation chart gives the effect of all parameters within the designed range, when the other parameters were kept constant on its middle value. Fig. 15 shows positive slopes (C-C and B-B) for solvent addition and reaction time with respect to the oil yield. Their influences get saturated at the higher range taken for study. However, the temperature and mixing intensities are optimum at their mid-range of the study.

3.2.2.1. Influence of solvent to biomass ratio and reaction time (dry basis)

The 3D plot and its corresponding contour plot are shown in Fig. 15. Chloroform/methanol solvent was an effective solvent for oil extraction on wet basis and the same was used here also. This solvent combination was successfully used for oil extraction from *Chlorella pyrenoidosa* dried biomass (1:1, v/v) [62] and for *Botryococcus branni* dried biomass at 2:1 (v/v) solvent biomass ratio [63]. Researchers have tested other solvent combinations hexane/isopropanol, dichloroethanol/ethanol, dichloroethane/ethanol, etc. and reported that chloroform/methanol as the best choice. When sufficient time (beyond 200 min) was provided, higher oil yield was obtained when the solvent biomass ratio was kept in the range between 9 and 14. Considering the interactive effect of other process parameters the best ratio predicted from the empirical model was 12:1.

The time for the reaction was another important parameter deciding the oil yield. In this work, the influence of time on oil yield was examined from 60 to 360 min (Fig. 16) and it was noted that the oil yield increases by time. However, the influence of time minimizes and gets saturated around 300 min. A minimum of 200 min was needed for better extraction and considering the interactive effect of other process parameters, the optimum time predicted by the model was 220 min.

3.2.2.2. Influence of extraction temperature and mixing intensity (dry basis)

The reaction temperature for the oil extraction process was varied from 55ºC to 75ºC. The oil yield increases from 9.1% at 55ºC to around 27.4% for dried biomass at 65ºC, since the temperature influences the dissolving power of the solvent (Fig. 16). However, further increase in temperature beyond the boiling point of the solvent leads to decrease in oil yield. The optimum predicted temperature for maximum yield was 68ºC. The effect of stirring speed on oil extraction is shown in Fig. 17. The influence of varying the

stirring speed on oil yield was very low with an F-value of only 9. Gentle stirring was important to ensure proper mixing for effective oil extraction. The optimum speed of the stirrer for the given condition was 600 rpm; varying the speed further away from this optimum value has resulted with negative effect on oil yield.

The optimum oil extraction process parameters on dry basis were statistically predicted from the empirical model to exploit the oil yield and are listed in Table 9.

Table 9
The predicted optimized process parameters to maximize the oil extraction on dry basis

Reaction temperature (°C)	Reaction time (min.)	Solvent biomass ratio (v/w)	Mixing intensity (rpm)	Oil yield (%)
68	220	12:1	600	27.5

The free fatty acid content was found out by the titration method through the acid value. The acid value of the oil was 1.02 mg KOH/g, which indicates the FFA content is fairy 0.51%. In the biodiesel conversion process, no need to consider the esterification process, since the acid value is less than 1 mg KOH/g. But most of the microalgae lipid contains more FFA value, which needs extra energy for further processing [64, 65].

3.3. Oil characterization

GC-MS was subjected to characterize the lipid to find the hydrocarbon compounds present in the composition. The fatty acid ranges from C11:0 to C26:0, which is dominated by monounsaturated fatty acids. Fig. 18 shows that the 32.39% of 11-Octadecenoic acid methyl ester and 55.88% of 13-Docosenoic acid methyl ester were dominated the monounsaturated fatty acid which offers the required oxidative stability and better combustion [66, 67, 68]. The existence of saturated and polyunsaturated fatty acids will limit the oxidation stability and makes the cold flow problems. Also, longer fatty acid carbon chains increase the cetane number to give better ignition and combustion characteristics [69, 70, 71]. Unsaturated fatty acid rises the fuel density and makes it less volatile, unfavourable to atomization, leads to carbon deposits in burning space. The greater acid value (> 1 mg KOH/g) of the biofuel destroys the rubber seals of the engine [72]. The inherent oxygen of hydrocarbons enhances the combustion properties and enables the decline in emission while using an alternative fuel in IC engine after conversion into biodiesel [73].

Conclusion

Finding a novel sustainable energy source is a tedious journey which needs insistent scientific work. The microalgae *Aphanothece halophytica* evidence a promising alternative energy resource which was mass propagated in open raceway ponds using an optimized medium. The biomass was harvested for the first time on 20th day (log max) and then on daily basis by employing organic neem plus flocculant which concentration was optimized as 0.002 ml/l for the maximum recovery of microalgal biomass within a

short span of 180 s. Oil extraction process parameters were optimized based on wet and dry basis using empirical models. The extraction time and solvent-biomass ratio are the major influencing factors along with temperature and mixing intensity. For wet biomass, high oil yield of 29.3% was achieved at 68ºC in 190 min at 9:1 solvent-biomass ratio, while stirring at 300 rpm. Similarly on dry basis, the maximum oil yield of around 27.5% was achieved when the solvent-biomass ratio was 12:1, while allowing for 220 min at 68ºC with a stirring speed of 600 rpm. The wet extraction procedure was more effective as it gives higher yield in less time with minimum solvent usage. The analysis of fatty acid profile by GC-MS shows the presence of 96.9% of monounsaturated fatty acid, 3.1% of saturated fatty acid and a negligible amount of polyunsaturated fatty acids to provide stability and good combustion phenomenon. The computed properties of algal biodiesel assure its usage in conventional diesel engines without any engine modification at less pollution.

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Figures

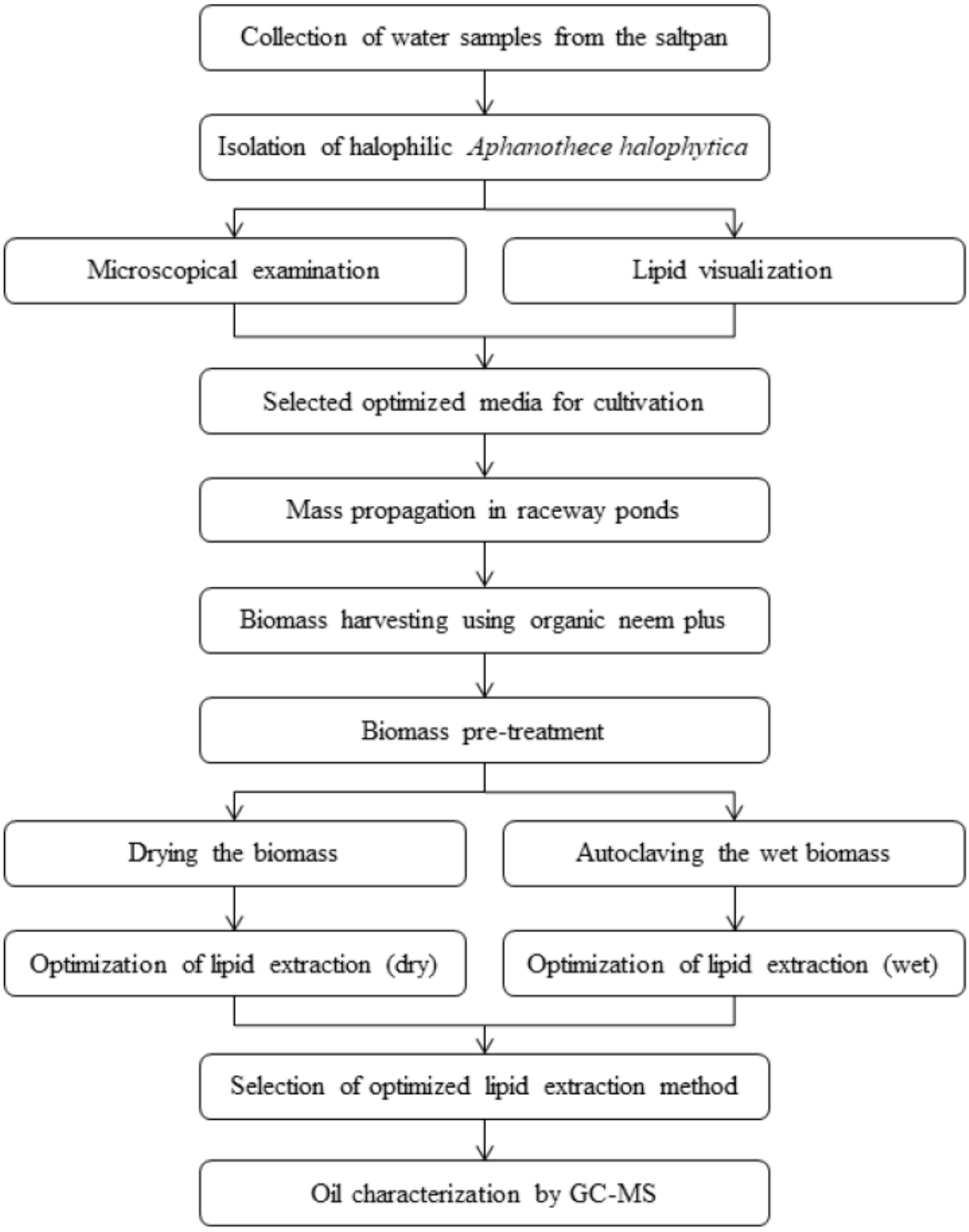

Figure 1

Methodology of the lipid extraction process from the microalgal strain

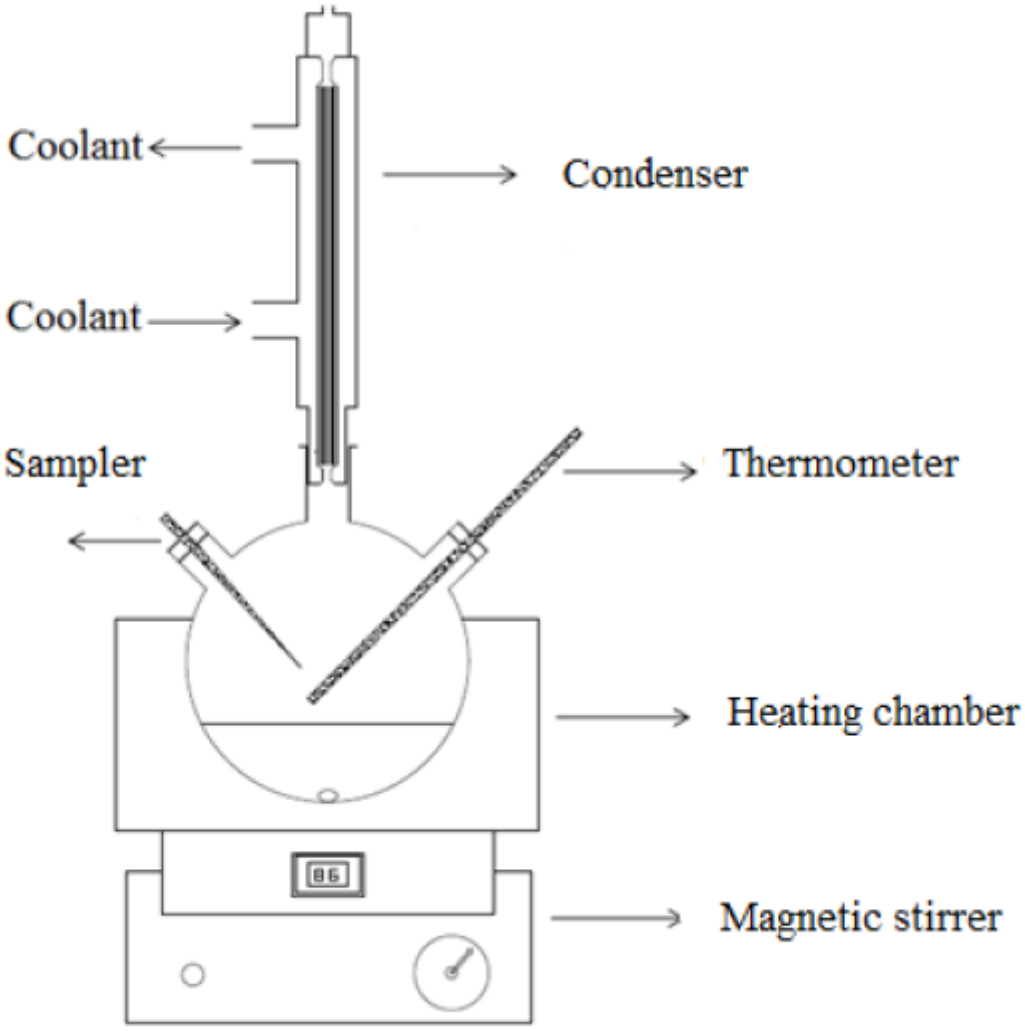

Figure 2

Block diagram of the batch mode lipid extraction setup

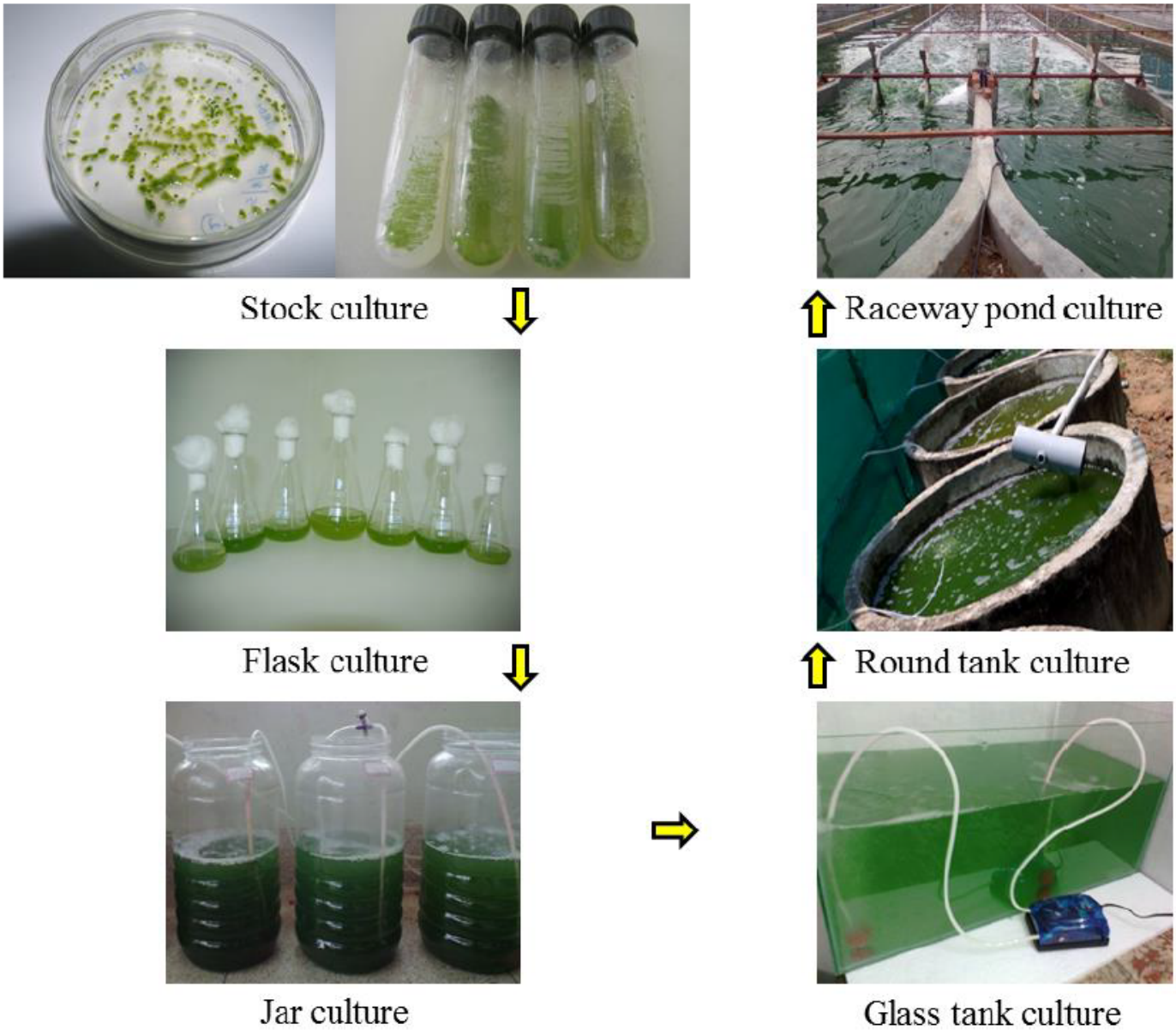

Figure 3

Mass propagation of microalgae from basic culture to raceway ponds

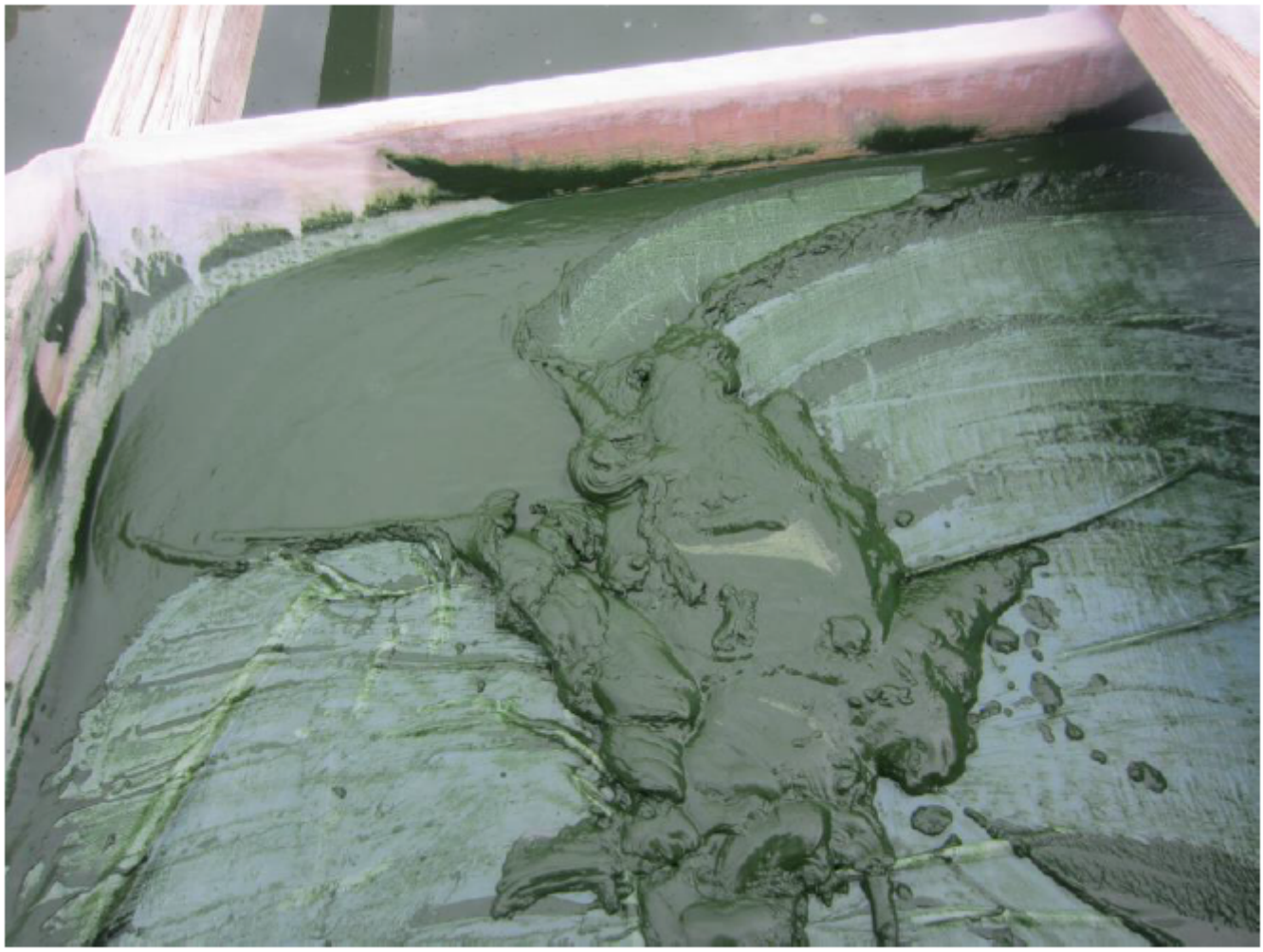

Figure 4

Harvested wet biomass for lipid extraction

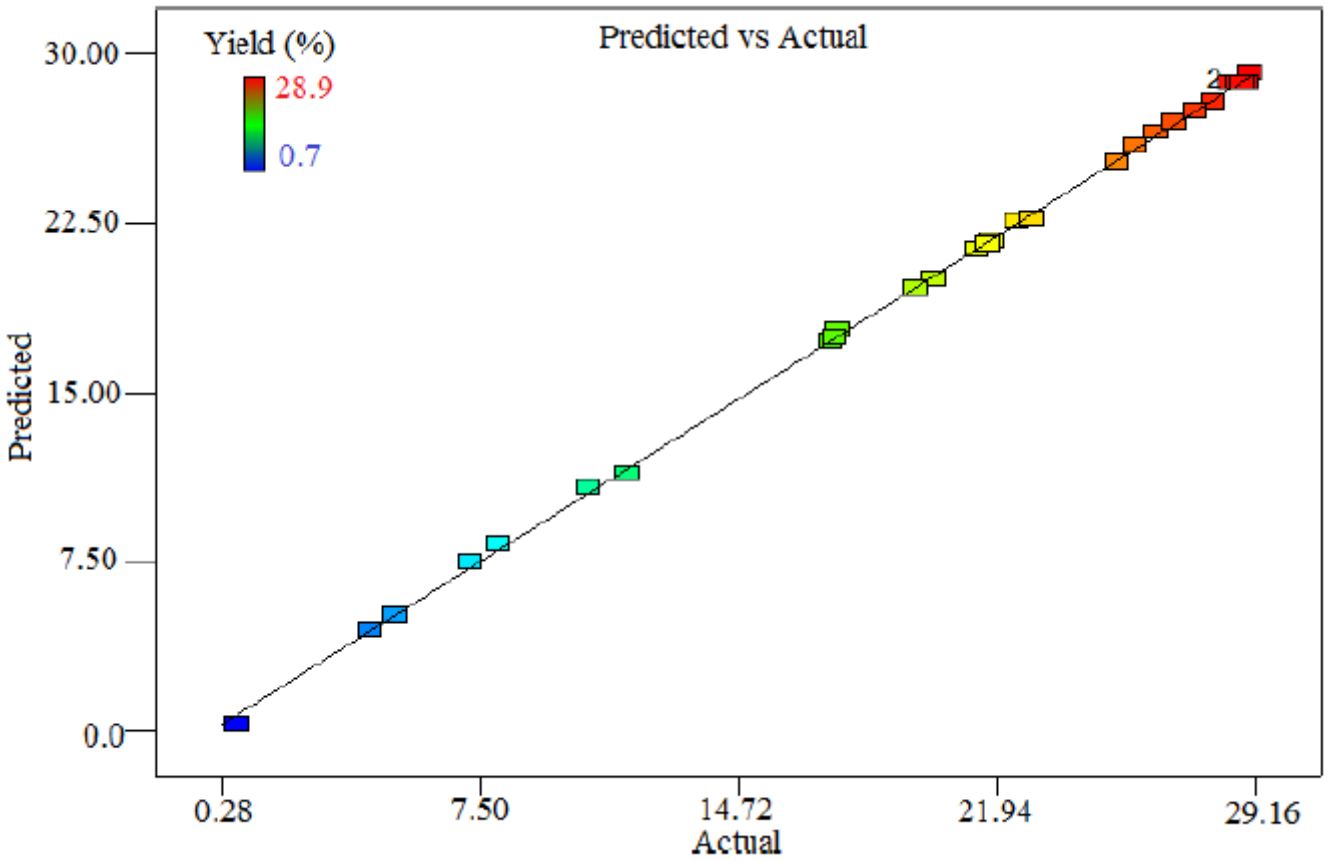

Figure 5

The plot of lipid yield by prediction vs actual yield for wet biomass

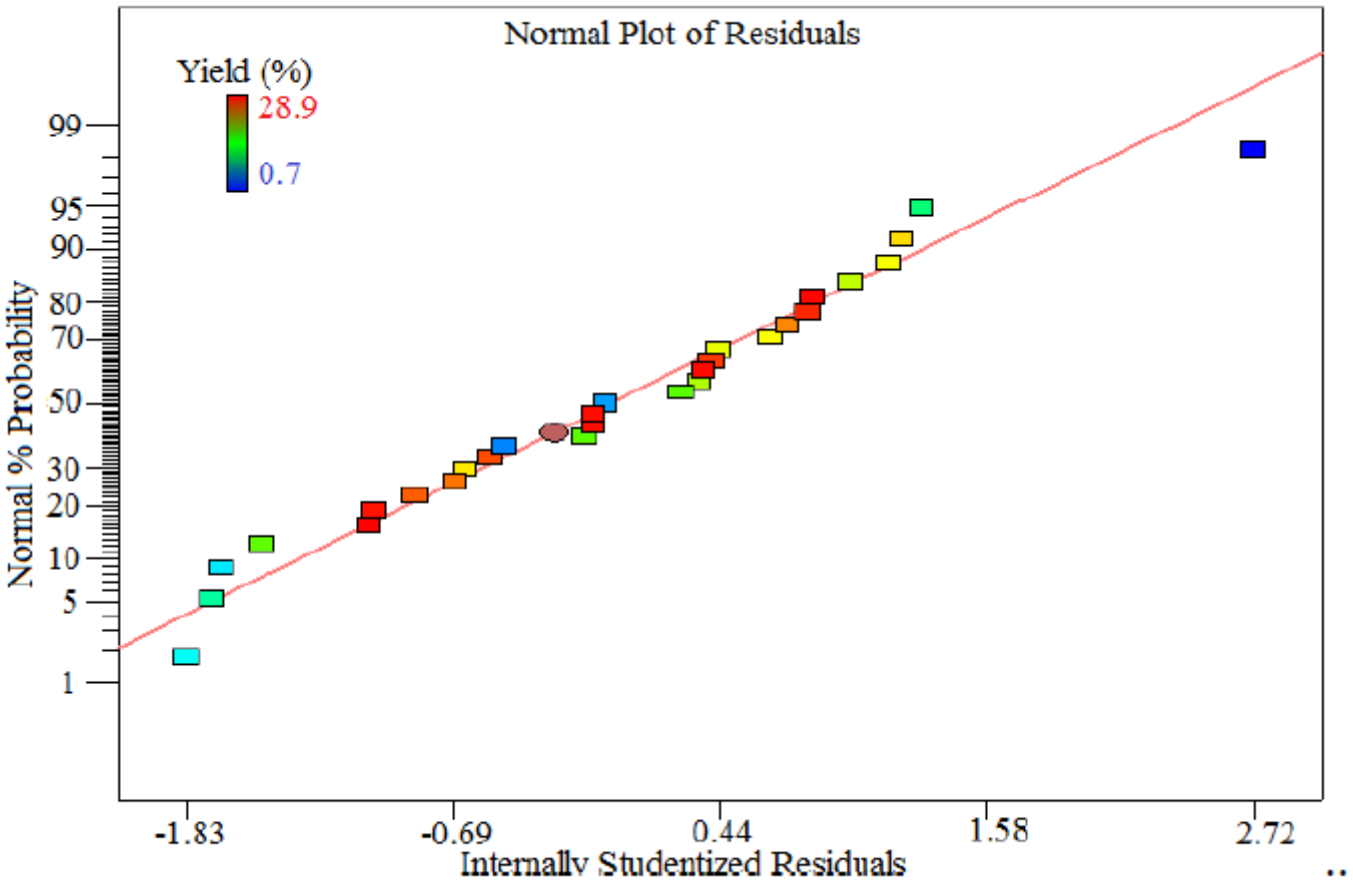

Figure 6

Normal probability plot of the residuals of the developed model to optimize the lipid extraction process on wet biomass basis

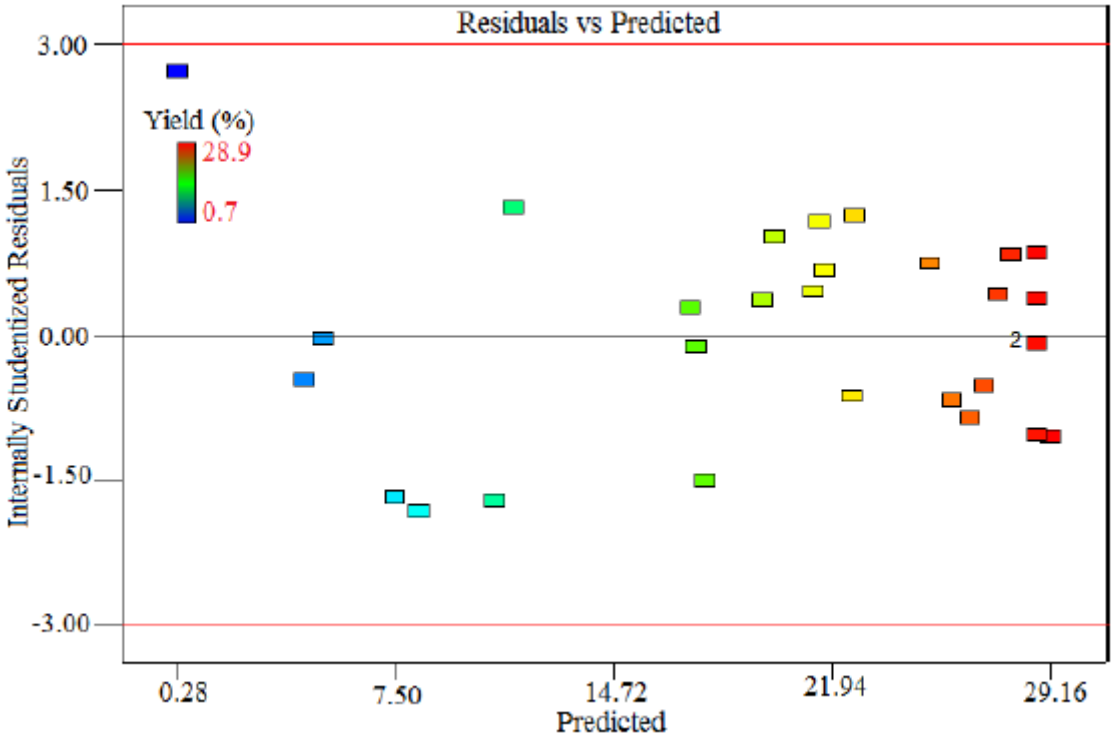

Figure 7

Plot of residual vs predicted response of the model to optimize the lipid extraction process on wet basis

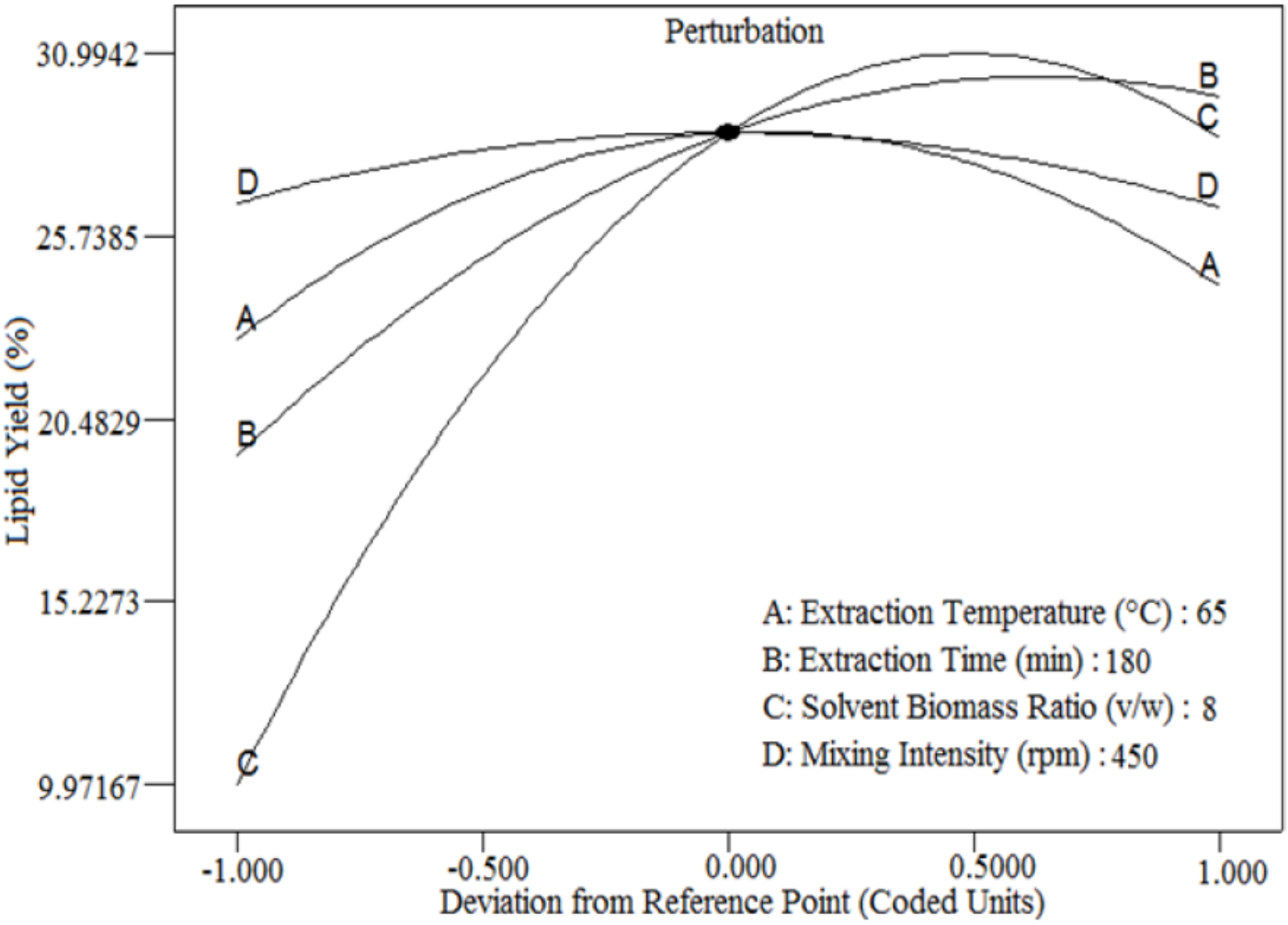

Figure 8

Perturbation chart to attain the influences of the lipid extraction process parameters on wet basis

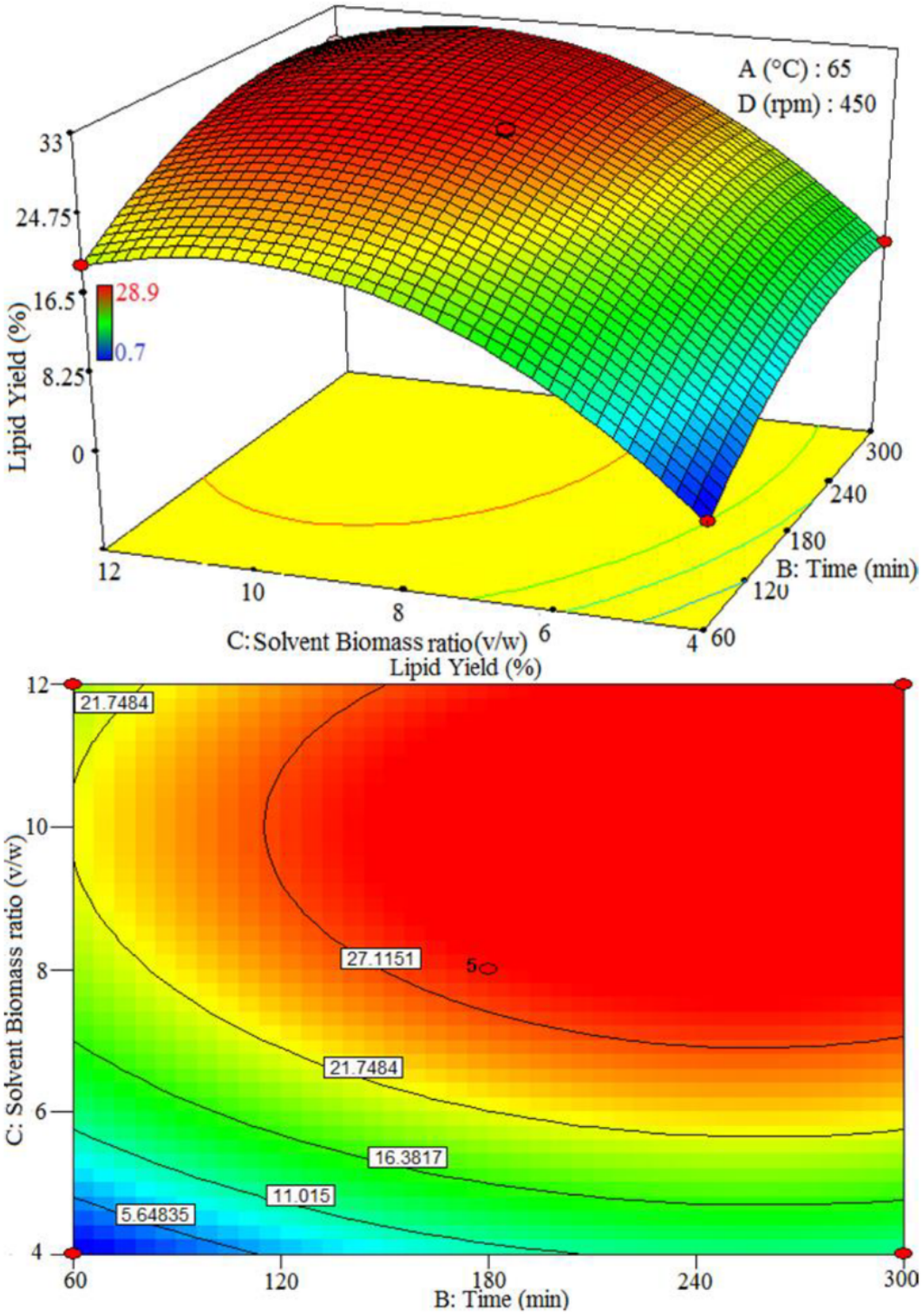

Figure 9

The 3D and contour plot of solvent biomass ratio and reaction time from the empirical model for maximizing the lipid yield from wet biomass

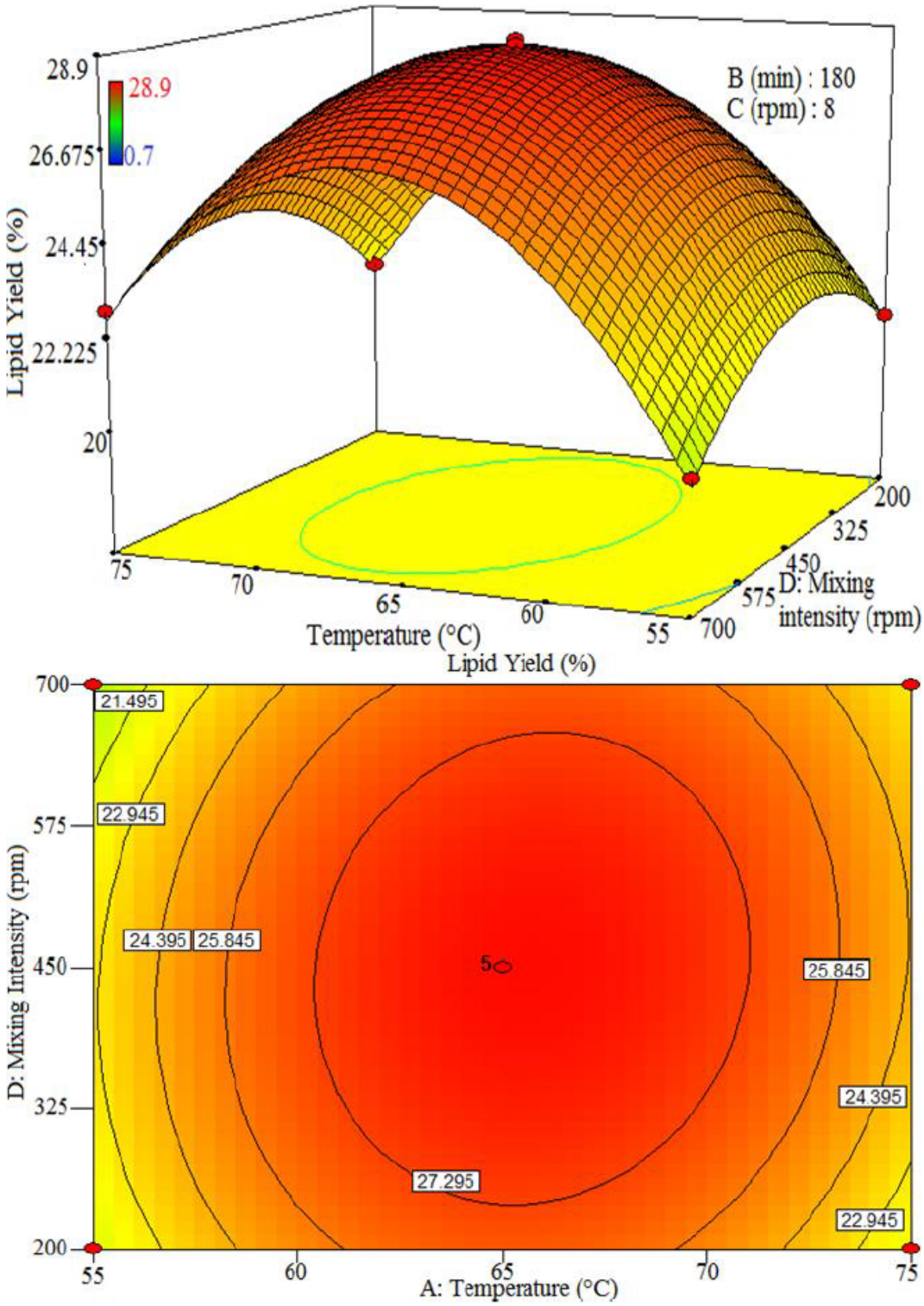

Figure 10

The 3D and contour plot of temperature and mixing intensity time from the empirical model for maximizing the lipid yield from wet biomass

Figure 11

Harvested dried biomass for lipid extraction

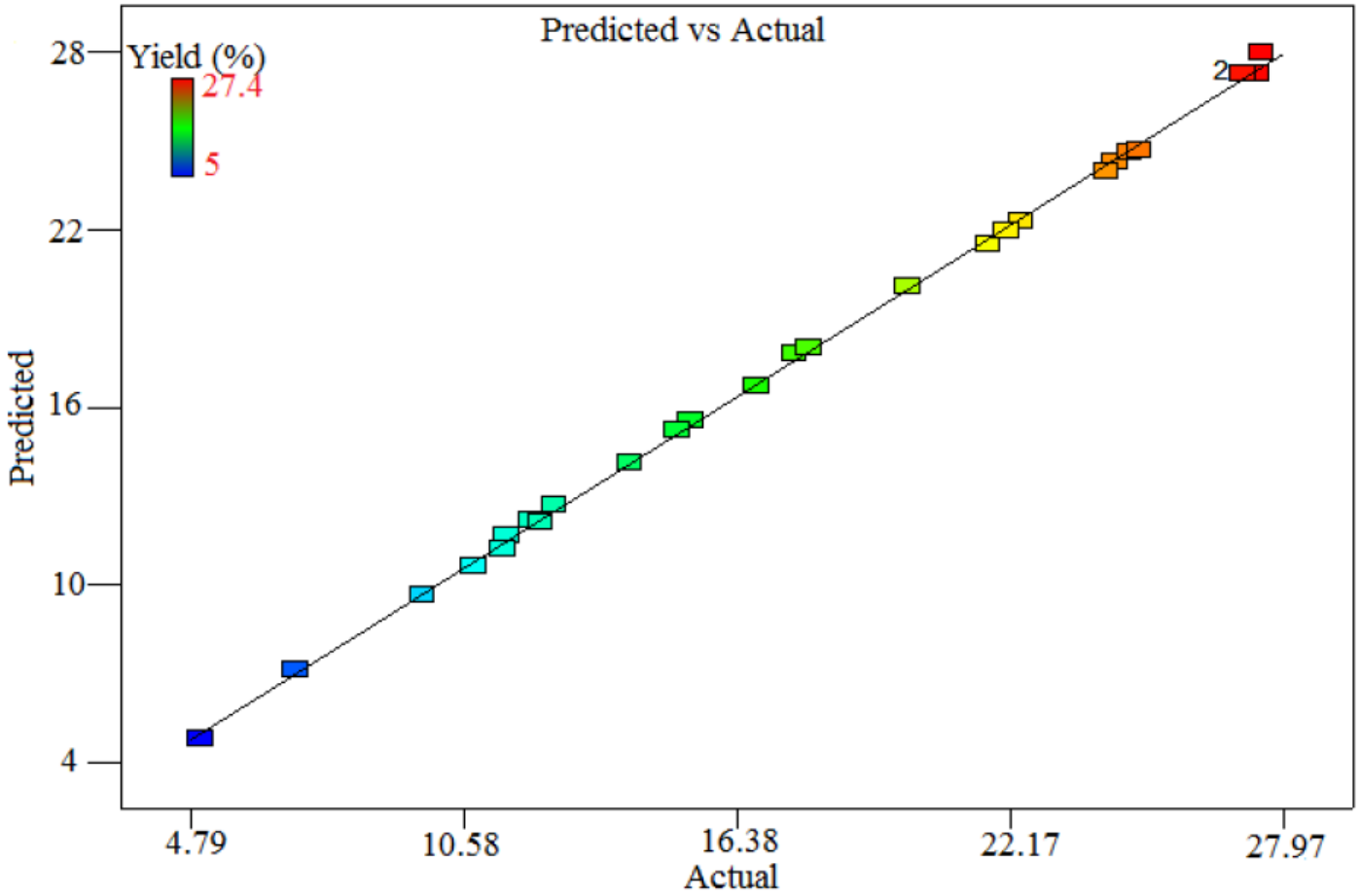

Figure 12

The plot of lipid yield by prediction vs actual yield for drybiomass

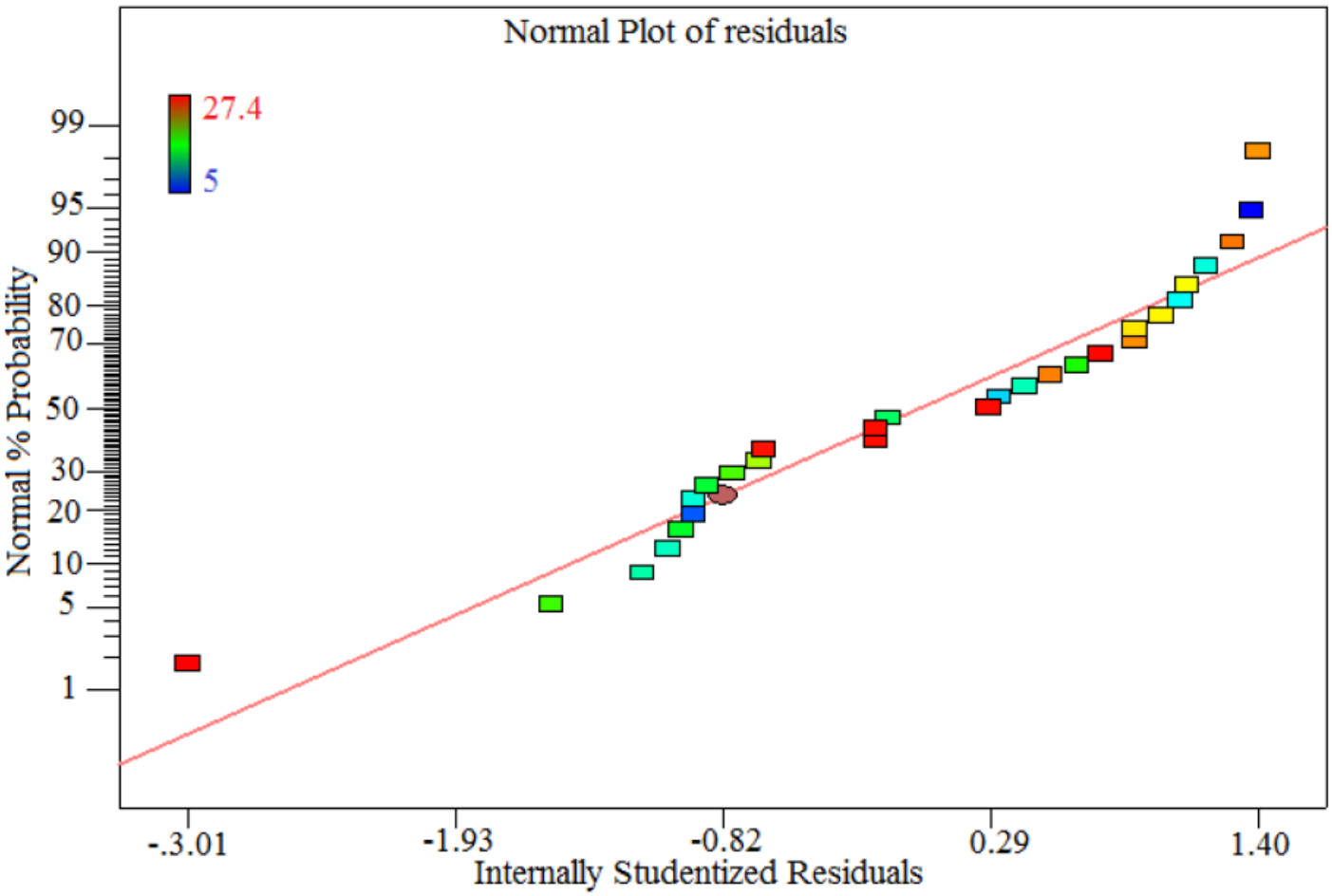

Figure 13

Normal probability plot of the residuals of the developed model to optimize the lipid extraction process on dry biomass basis

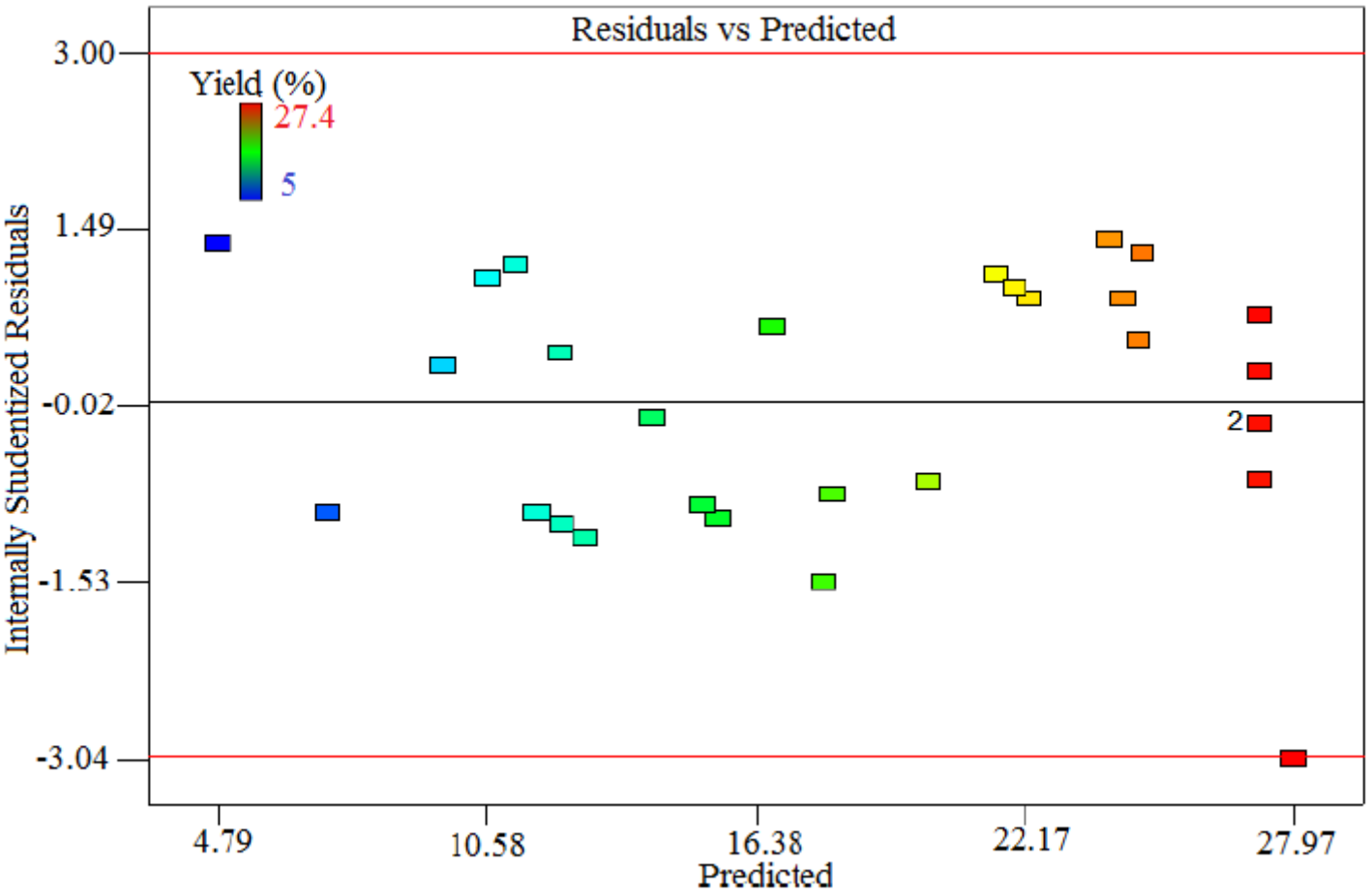

Figure 14

Plot of residual vs predicted response of the model to optimize the lipid extraction process on dry basis

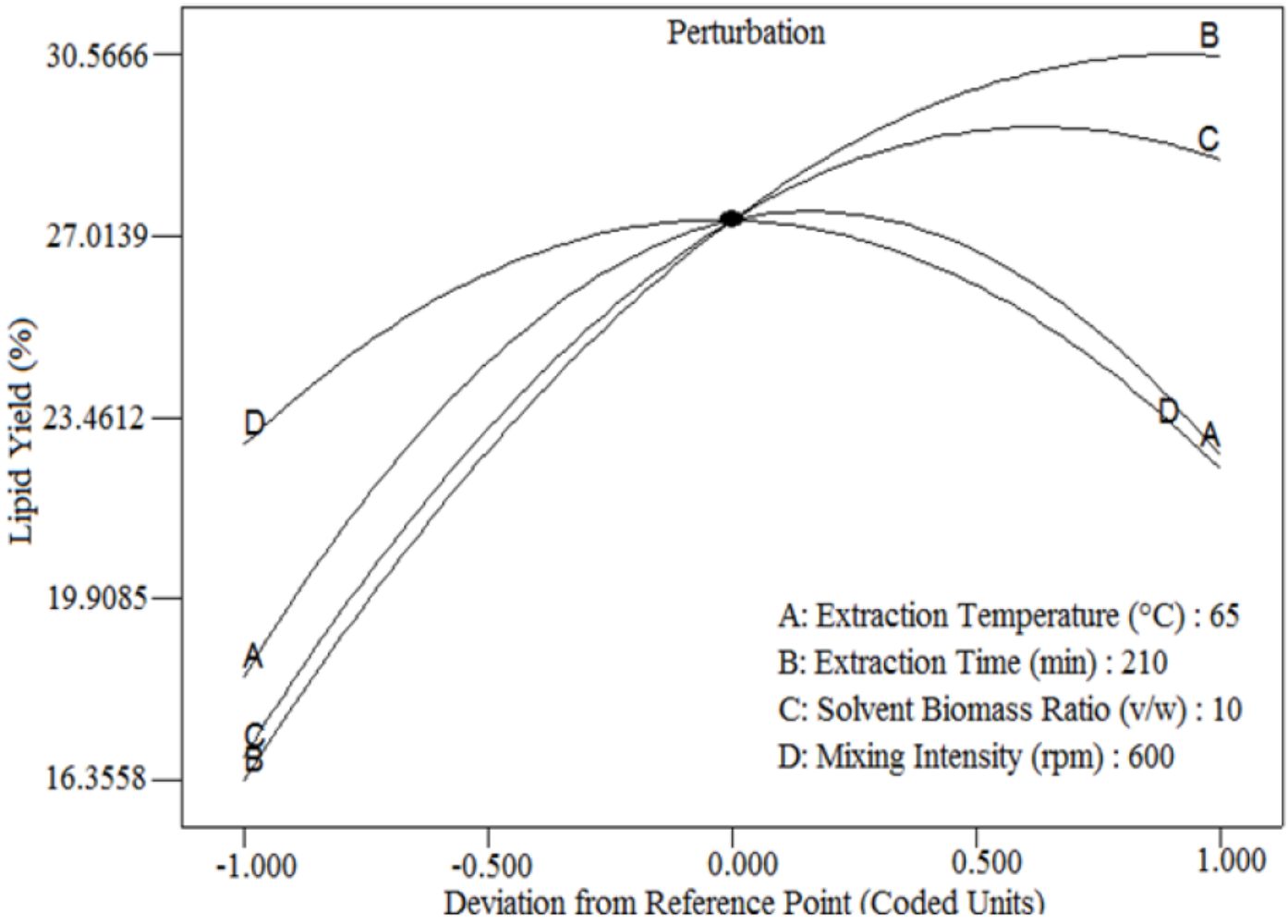

Figure 15

Perturbation chart to attain the influences of the lipid extraction process parameters on dry basis

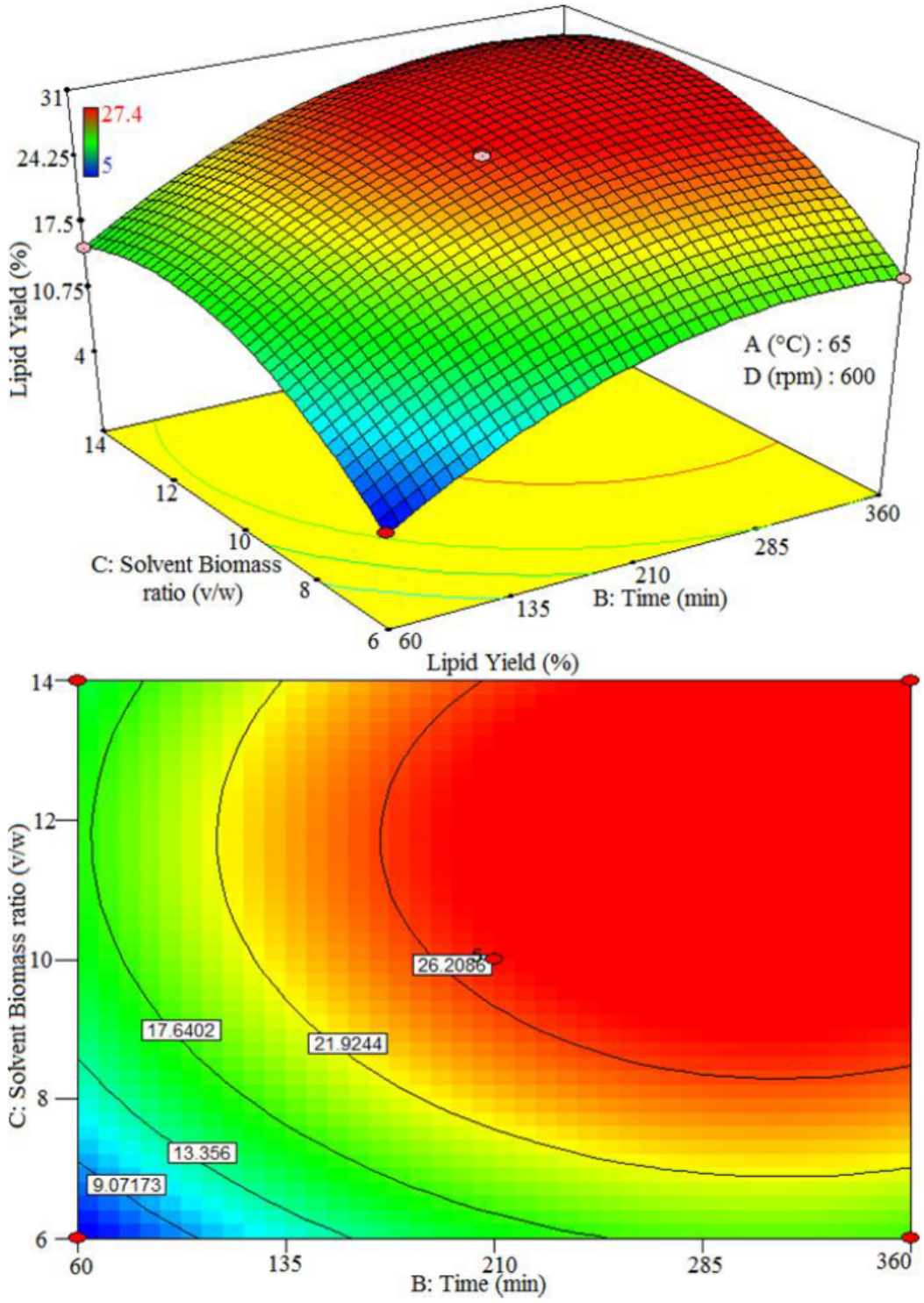

Figure 16

The 3D and contour plot of solvent biomass ratio and reaction time from the empirical model for maximizing the lipid yield from dry biomass

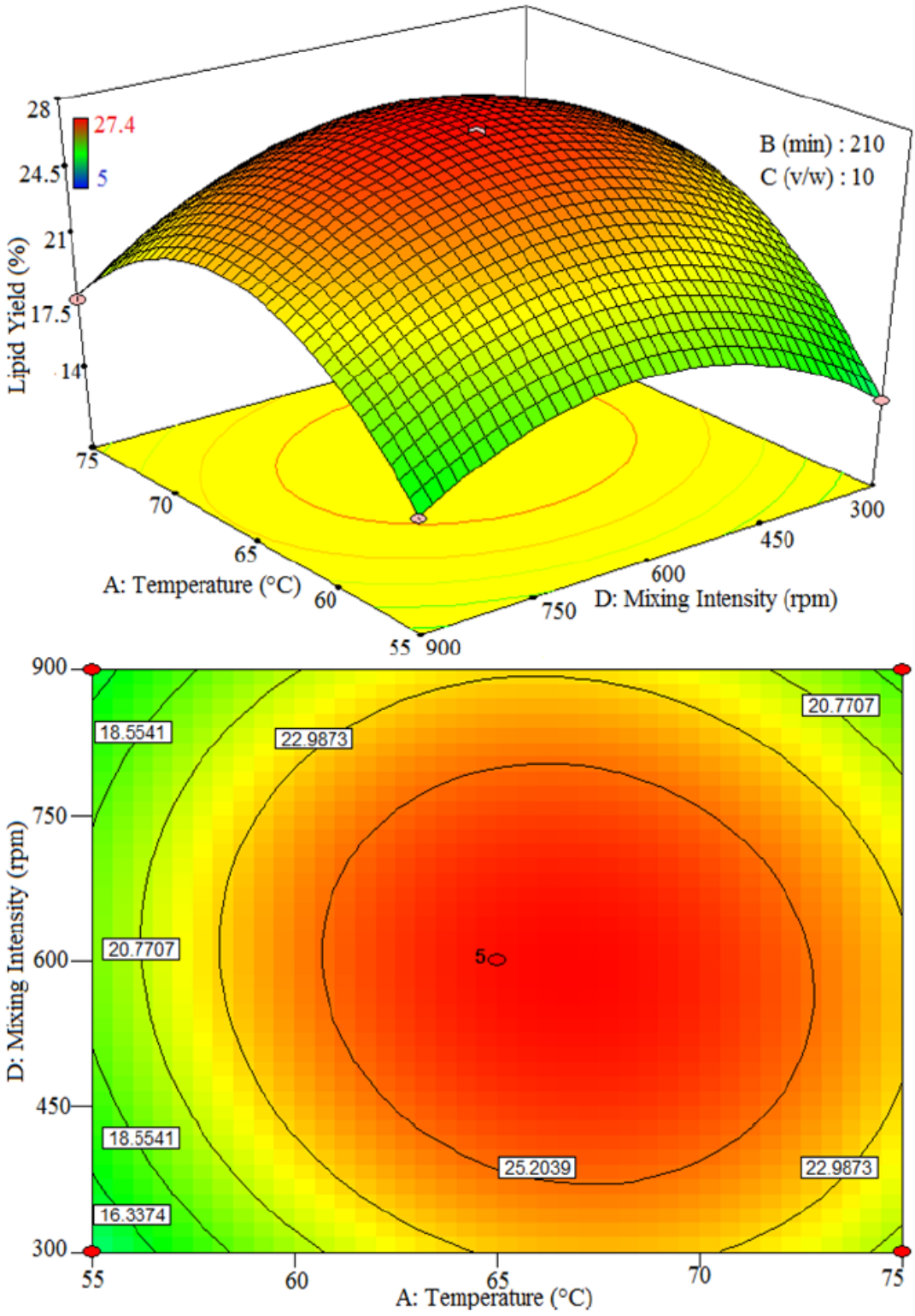

Figure 17

The 3D and contour plot of temperature and mixing intensity time from the empirical model for maximizing the lipid yield from dry biomass

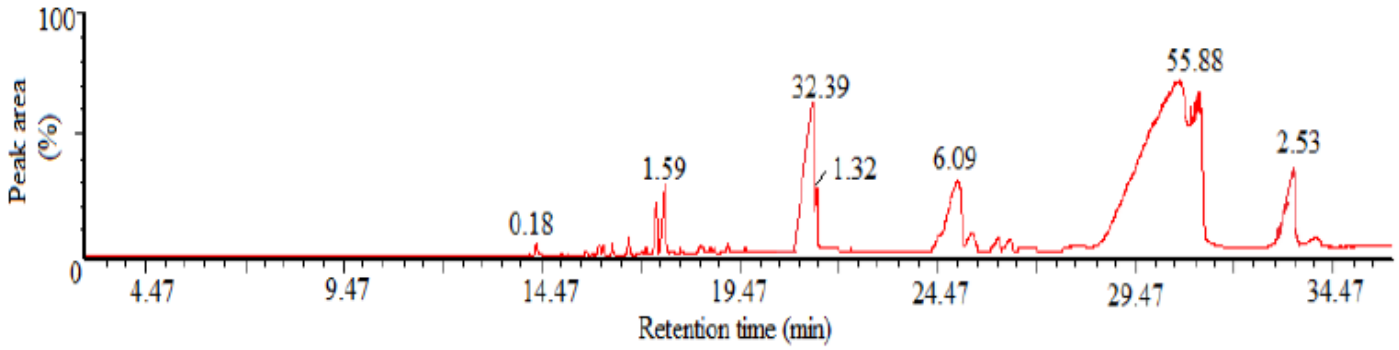

Figure 18

Graphical view of the fatty acid profile composition