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Development of a Selective Method for Metabolites Extraction from Microalgae Biomass

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Abstract

Background: The production of biofuel and high value products from microalgae exhibits difficulties that have been widely studied to develop viable, efficient and economic methods for recovering metabolites. **Objectives**: This work is focused on evaluating experimental methods to obtain carbohydrates, proteins and lipids by varying process variables (solvent concentration, temperature, biomass/solvent ratio and moisture content). **Methods/Analysis**: Carbohydrate and proteins were extracted by acid and alkaline hydrolysis to study the effect of biomass moisture on recovery of these metabolites. Lipids were obtained using hexane and methanol-chloroform methods and its quantification was performed by gravimetric analysis. Findings: It was found that 41.96% and 49.77% of carbohydrates were recovered from *C. vulgaris* using biomass without thermal pretreatment by acid and alkaline hydrolysis, respectively. Regarding to lipid extraction, hexane was used as solvent for recovering 18.22% of lipids from *C. vulgaris.* In addition, results suggested that dehydrating biomass at 105°C reduces recovery of high value products. **Novelty/Improvement:** This study proposes a selective method for extracting metabolites, which enhances efficiency of recovery when is carried out under suitable conditions of biomass moisture, time and solvent volume.

Keywords: Carbohydrates, Flocculation, Lipids, Microalgae, Proteins

1. Introduction

Microalgae have been widely studied in last few decades as source of high value products in food, energy and pharmaceutical industry[1]. They are fast growing photosynthetic organisms which can be found flourishing in surrounding environment with minimal nutrient supplements[2,3]. The ability of microalgae to survive or proliferate over a wide range of environmental conditions results in the production of an array of many secondary metabolites, which are of considerable value in biotechnology fields including aquaculture, health and food[4]. Microalgae have a very broad biodiversity, it is estimated that there are about 200,000-800,000 species of microalgae, from which about 50,000 species are officially known and described[5]. Among these microalgae, *Chlorella* is currently domi-

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nating the microalgal market[6]. Being a natural source of highly interesting biologically active compounds with positive health effects, microalgae produce a range of functional ingredients including polyunsaturated fatty acids, polysaccharides, natural pigments, essential minerals, vitamins, essential amino acids, and enzymes, as well as bioactive molecules[7]. Most of the functional components are associated to lipid (oil), protein and carbohydrate in microalgae biomass[8]. Lipid content in microalgae depends on microalgae species. Its content can be up to 40% for some engineered microalgae[9]. Algal carbohydrates are investigated for many potential applications, for example renewable energies or human health products[10]. Proteins from microalgae are high in quality, comparable to conventional vegetable proteins[11]. Development of extraction techniques for microalgae components has become a field of growing interest for the scientific community. Because of the great economic stakes for high value products from microalgae, many studies have aimed to find efficient and economic ways to recover them[12]. This work aims to determine a selective method for extracting proteins, carbohydrates and lipids from *C. vulgaris* through evaluating the effect of thermal pretreatment on efficiency of metabolites recovery and using acid/alkaline hydrolysis in proteins and carbohydrates recovery and hexane/methanol-chloroform solvents in lipid extraction.

2. Material and Methods

2.1 Culture Methods

Chlorella vulgaris UTEX 1803 was obtained from the strain collection at University of Texas (Texas, USA), which was cultivated in Bold Basal medium. Main components of this medium are (mg/L): $NaNO_3$ (2.94), $MgSO_4.7H_2O$ ($3.04x10^{-1}$) NaCl ($4.28x10^{-1}$), K_2HPO_4 ($4.31x10^{-1}$), KH_2PO_4 (1.29), $CaCl_2.2H_2O$ ($1.70\ x10^{-1}$), $ZnSO_4.7H_2O$ ($3.07\ x10^{-2}$), EDTA ($1.71x10^{-1}$), KOH ($5.53\ x10^{-1}$) and $FeSO_4.7H_2O$ ($1.79\ x10^{-2}$). The microalgae was kept growing during 15 days in 20 L rectangular glass reactor. The reactor was coupled to a bubble aeration system for air injection and light-dark cycle 12:12 h.

2.2 Thermal Pretreatment of Biomass

Biomass was centrifuged at 3400 rpm during 15 minutes in order to separate liquid-solid mixture. The concentrated biomass (solid phase) was thermally pretreated and subjected to acid or alkaline hydrolysis. In addition, a control test was performed without thermal pretreatment.

2.3 Determination of Moisture Content

The moisture content of biomass on dry basis was determined by gravimetric analysis. A representative sample of biomass after thermal pre-treatment was selected and subjected to 105 ºC during 17 hours. This test was performed in triplicate and the average value was taken as reference for quantifying recovery of carbohydrates and proteins in dry basis.

2.4 Evaluation of the Effect of Moisture Content on Proteins and Carbohydrates Extraction using Two Routes of Hydrolysis

2.4.1 Acid Hydrolysis

This hydrolysis was based on modified procedure from[13] by adding 5 g of pretreated biomass and 50 ml HCl (0.5 M). The mixture was continuously stirred at 500 rpm during 2 hours. Then, KOH solution was gradually added to neutralize reaction products. Separation and filtration of liquor (high content of carbohydrates) and hydrolyzed biomass was performed.

2.4.2 Alkaline Hydrolysis

Sodium hydroxide is a common reagent to be used for alkaline pretreatment[14], hence 30 ml of NaOH (3.67 M) was mixed with 5 g of pretreated biomass. Then, it was heated during 20 minutes at 55ºC. After finalizing the reaction, pH was adjusted to 7.5-8.5 and solid-liquid separation was carried out in order to obtain liquor (rich in carbohydrates and proteins) and residual biomass.

2.4.3 *Quantification of Carbohydrates*

The carbohydrate content in liquor from acid hydrolysis was determined using phenol-sulfuric acid colorimetric method[15]. The sample (1 ml) was added to 0.5 ml of 5% phenol and 2.5 ml of 95% sulfuric acid. After revealing the coloration, absorbance measurement was performed at 485 nm (wavelength). The glucose concentration in the extract was quantified by the standardized concentration curve from the analytical grade reagent: D (+) Glucose.

2.4.4 *Quantification of Proteins*

Protein content in the extract was determined by method described[16]. This procedure consisted of mixing 1 ml of simple and 1.4 ml of Lowry solution. The solution was homogenized during 5 minutes and 0.2 ml of Folin-water solution was added. When the coloration was revealed, protein content was determined by calibration curve, previously standardized from the analytical grade albumin reagent. The measurements were performed at 750 nm wavelength using Spectroquant Pharo 300 spectrophotometer (Merck).

2.4.5 *Experimental Design*

To evaluate the effect of moisture in pretreated biomass on carbohydrate and protein recovery, nine tests were carried out for each route varying time and temperature of drying as summarized Table 1. The experimental design was performed using STATISTICA 7.0 software.

2.5 Lipid Extraction using Hexane and Mixture of Methanol-Chloroform as Solvent

Biomass from *C. vulgaris* was concentrated by centrifugation and divided in two portions, one of them was sent to a dryer at 105ºC during 17 hours. The remaining portion (wet biomass) was subjected to extraction methods. The lipid contents were quantified by gravimetric analysis in extracted solution.

Table 1. Experimental design for extracting proteins and carbohydrates from pretreated biomass

Test	Temperature (ºC)	Time (h)
1	33	18
2	45	12
3	45	24
4	75	10
5	75	18
6	75	26
7	105	12
8	105	24
9	117	18

2.5.1 Hexane Method

Biomass sample was mixed with 1 ml of H_2SO_4 solution (1 M) in falcon tube. The mixture was heated at 90ºC during 30 minutes. Then, 1 ml of NaOH (5 M) was added and heated at 90ºC during 30 minutes. The solution was centrifuged at 3400 rpm during 15 minutes. Hexane was used as solvent with different volumes (2.36, 3.4, 5, 6.6 and 7.67 ml) in precipitated phase and this sample was heated during 15 minutes at five temperatures (13, 25, 42, 60 and 71 ºC).

2.5.2 Methanol-Chloroform Method

Biomass sample was added to 5.7 ml of Bligh & Dyer solution in falcon tube. The mixture was homogenized during 10 minutes. Then, it was centrifuged at 3400 rpm during 15 minutes. This procedure was repeated with solid phase after centrifugation. The liquid phase was mixed with 1.3 ml of distilled water and 3 ml of chloroform.

2.5.3 Experimental Design

In order to extract lipid from biomass both routes of hexane and methanol-chloroform were carried out using

Table 2. Experimental design for lipid extraction using hexane

Test	Biomass (mg)	Temperature (°C)	Volume of solvent (ml)
A	290,33	42,50	5
B	625	42,50	5
C	625	42,50	5
D	625	42,50	5
E	959,66	42,50	5
F	625	42,50	7,67
G	625	42,50	2,32
H	825	60	6,60
I	425	60	3,40
J	425	60	6,60
K	825	60	3,40
L	825	25	3,40

Table 2 Continued

Test	Biomass (mg)	Temperature (°C)	Volume of solvent (ml)
M	825	25	6,60
N	425	25	6,60
O	425	25	3,40
P	625	71,78	5
Q	625	13,21	5

Table 3. Experimental design for lipid extraction using metanol-chloroform

Test	Biomass/Solvent (g/L)	Methanol (ml)	Chloroform (ml)	H_2O (ml)
A	5	947	30	23
B	15	947	30	23
C	5	699	22	279
D	15	699	22	279
E	5	526	263	211
F	15	526	263	211
G	5	127	63	810
H	15	127	63	810
I	20	526	263	211
J	10	526	263	211
K	5	526	263	211

Table 3 Continued

Test	Biomass/Solvent (g/L)	Methanol (ml)	Chloroform (ml)	H_2O (ml)
L	15	526	263	211
M	5	65	519	416
N	15	65	519	416
O	5	656	328	16
P	15	656	328	16
Q	5	106	851	43
R	15	106	851	43
S	10	841	88	71
T	10	0	556	444
U	10	667	333	0
V	10	294	147	559
W	10	265	629	106

central composite design 3^3 and 4^3, respectively, as is summarized in Table 2 & 3.

3. Results and Discussion

3.1 Carbohydrate Extraction using Acid Hydrolysis

Table 4 displays moisture values obtained in each experiment and yield of carbohydrate recovery, which was calculated on dry basis.

Figure 1 shows the results of carbohydrates recovery from biomass (*C. vulgaris*) subjected to thermal pretreatment, under conditions of T (° C) and t (h) according to experimental design. It was found that carbohydrate recovery yield of 41.96% was obtained using biomass with high moisture content (72.55%) obtained during the control test (without thermal pretreatment). This result suggested that large investments in equipment for drying are not required to extract the highest amount of fermentable sugars; hence, the reduction of costs increases the suitability for scaling up this method to pilot plant.

Table 4. Results of carbohydrate extraction using acid hydrolysis

Test	C	1	2	3	4	5	6	7	8	9
Conditions [T (°C) / t (h)]	N.A	33/18	45/12	45/24	75/9.5	75/18	75/26.5	105/12	105/24	117/18
Moisture (%)	72.55	66.70	20.69	12.30	10.57	8.21	6.55	3.33	0.20	0.50
Carbohydrate recovery (%)	41.96	19.14	9.85	16.07	17.32	7.93	6.94	10.18	10.26	8.68

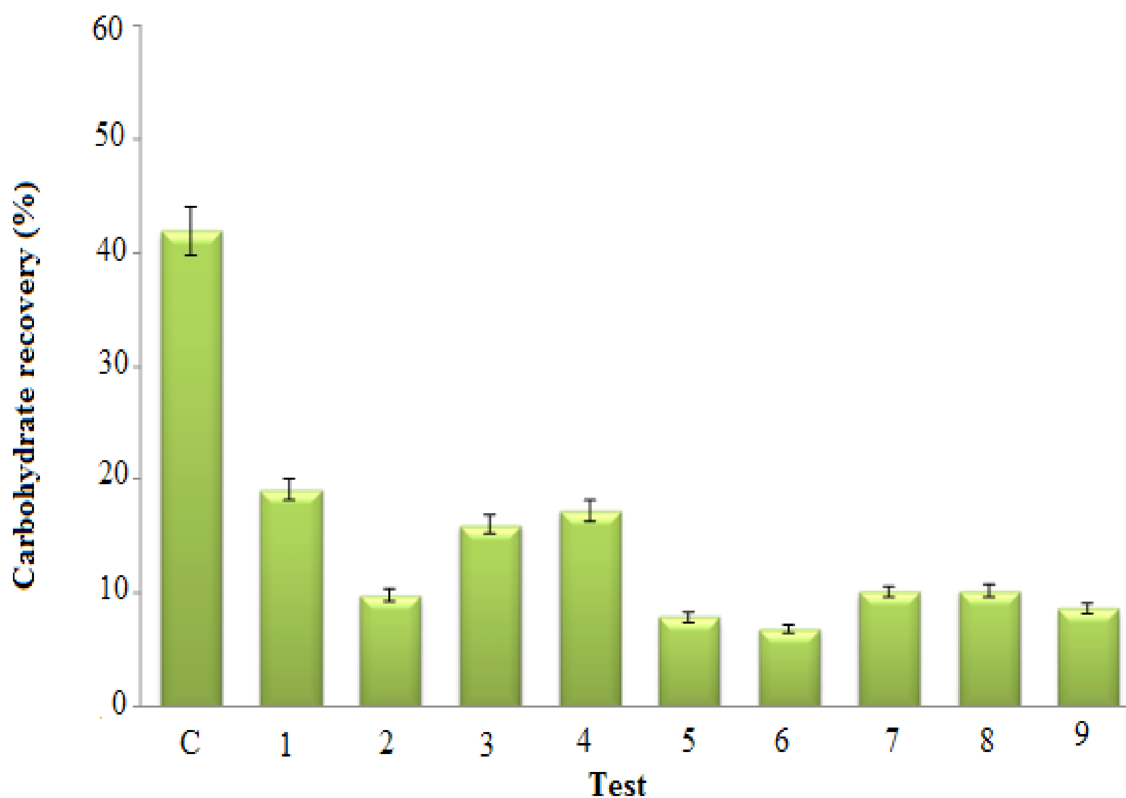

Figure 1. Carbohydrate recovery from microalgae biomass using acid hydrolysis.

The amount of extracted carbohydrates (8.68%) was lower in pretreated biomass at higher temperature during longer times (117 °C/18h) in comparison to control test (41.96%). According to[17], the excess of temperature can degrade lignocellulosic materials, breaking down some carbohydrates molecules. In[18] also found that water content decreases the action of hexane during the extraction of hydrocarbons from *Botryococcus braunni* due to solvent polarity. Therefore, if the solvent is immiscible in aqueous phase, moisture content in biomass can inhibit hydrolysis or extraction process. The results of this work indicated that water enhances the miscible nature of extractive

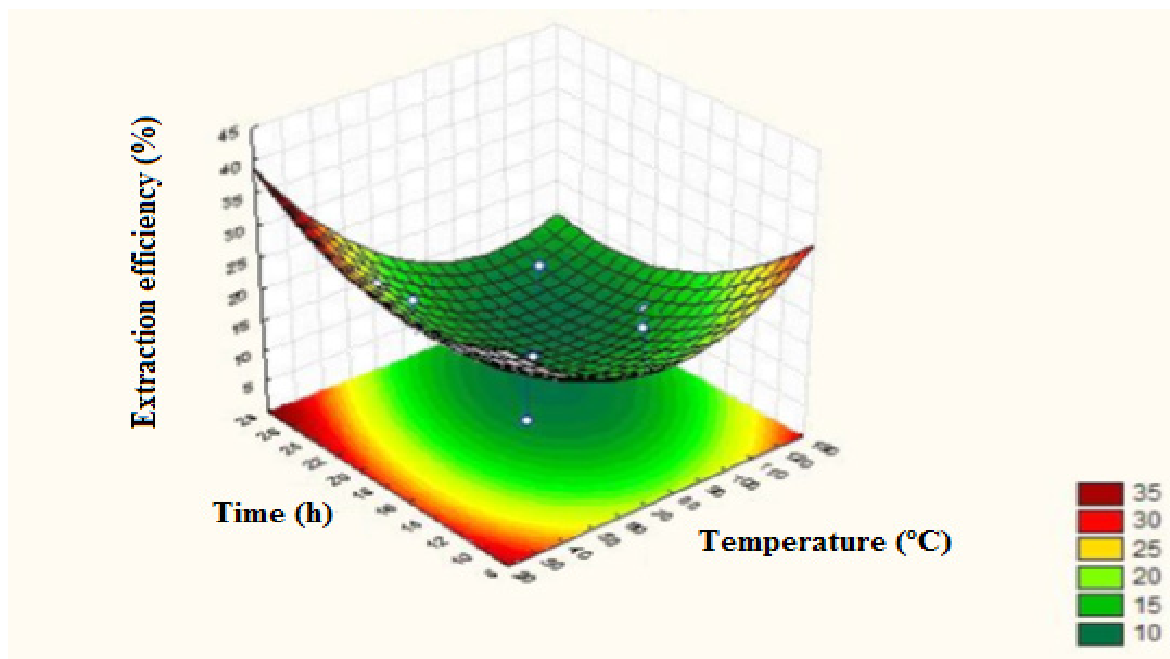

Figure 2. Response surface of extraction efficiency using acid hydrolysis.

agent (HCl) and increases its efficiency. It is important to highlight that proteins were impossible to extract due to degradation of substrate destroying a large part of quantifiable amino acids. In order to recover proteins by acid hydrolysis, it is required to control the reaction by enzymatic action and other variable of influences such as pH

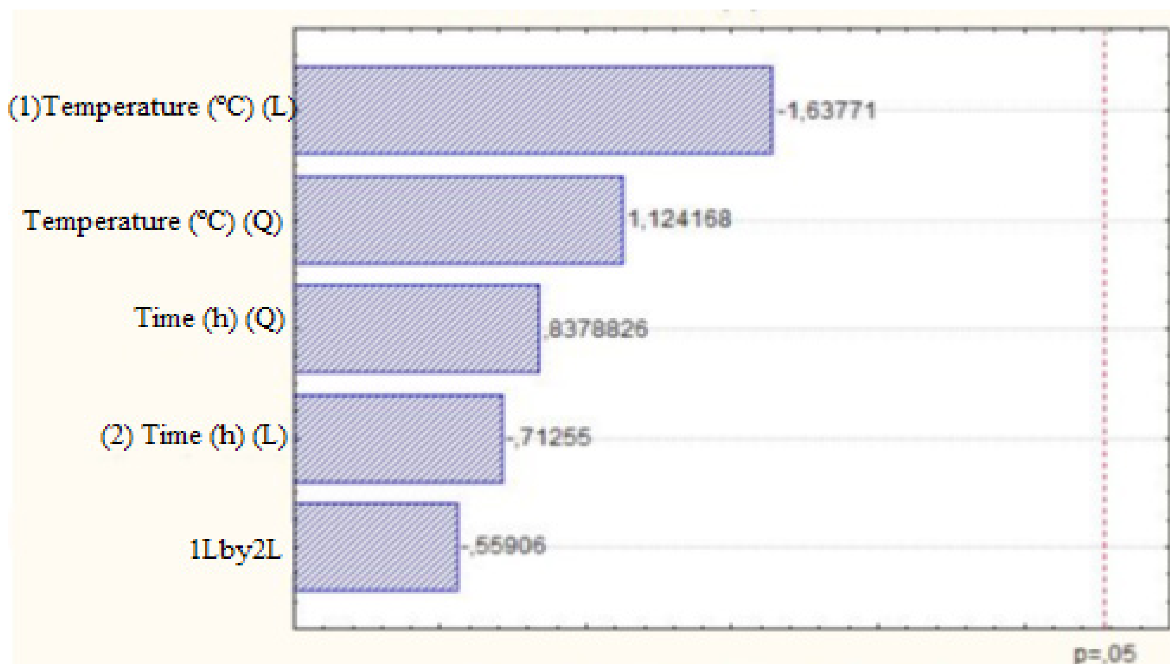

Figure 3. Pareto diagram for carbohydrate extraction using alkaline hydrolysis.

and temperature. Hence, the extraction of metabolites by acid hydrolysis is limited to obtaining fermentable sugars.

The effect of thermal pre-treatment (biomass moisture) on recovery carbohydrates from *C. vulgaris* was analyzed by Statistica 7.0 software. Figure 2 shows the response surface of process efficiency as function of temperature and time. The process of carbohydrate recovery is more efficient when biomass has not been exposed to drying under high temperatures. According to these results, it is inferred that recovery yield increases under temperature less than 20ºC and time between 0-24 hours. In order to verify the influence of both variables on carbohydrate recovery, Pareto diagram was performed and shown in Figure 3. It is observed that temperature and time did not affect significantly carbohydrate extraction process. The statistical analysis demonstrates that fermentable sugars production from *C. vulgaris* is not highly dependent on its moisture content.

3.2 Carbohydrate and Protein Extraction using Alkaline Hydrolysis

Table 5 summarized moisture values obtained for each experiment and yield of carbohydrates and proteins recovery, which were calculated on dry basis.

The results for proteins and carbohydrate extraction from biomass (*C. vulgaris*) subjected to thermal pretreatment under specific conditions of T (° C) and t (h) are presented in Figure 4. It is observed that 71.51% and 49.77% of protein and carbohydrate are recovered using biomass without thermal pretreatment, respectively. However, Lam & Lee[19] claim that dehydrating biomass at high temperature facilitates the breakdown of cell wall. While, other authors indicate that drying process can cause significant deterioration of molecules and decrease efficiency of extraction[20]. In[21] propose to use biomass with high content of moisture in order to reduce the cost generated by thermal pre-treatment stage. At laboratory scale, dehydrating biomass represents energy consumption up to 1.6 kW/h, using biomass with high moisture content is a viable alternative for producing metabolites.

In contrast to results shown in Figure 3, In[22] recovered up to 74.2% of carbohydrates from dehydrated biomass (*Chlorococcuminfusionum*) using an alkaline solution (NaOH) 0.75% (w/v) at 120 °C during 30 minutes. Regarding to proteins, alkaline hydrolysis at 40 °C

Table 5. Results of carbohydrate extraction using alkaline hydrolysis

Test	C	1	2	3	4	5	6	7	8	9
Conditions [T (°C) / t (h)]	N.A	33/18	45/12	45/24	75/10	75/18	75/26	105/12	105/24	117/18
Moisture (%)	71.05	11.11	8.06	8.21	4.07	2.23	1.60	1.32	0.50	0.14
Carbohydrate recovery (%)	49.77	36.40	42.18	42.09	22.41	29.43	44.04	32.39	29.65	27.71
Proteinrecovery (%)	70.51	44.01	39.15	49.75	52.95	54.14	53.65	57.84	60.55	40.18

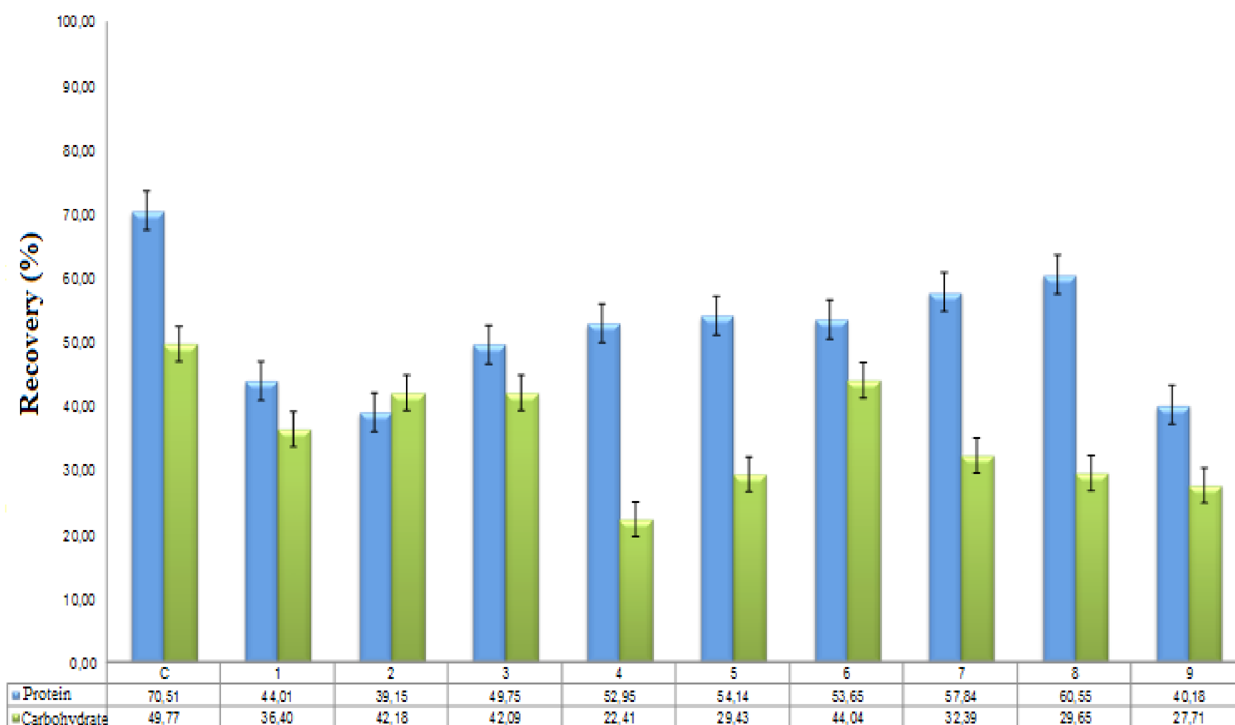

Figure 4. Carbohydrate and protein recovery from microalgae biomass using alkaline hydrolysis.

studied[23] achieved recovery less than 20% in *Chlorella vulgaris* in comparison to different cell disruption treatments such as: high pressure, ultrasound and mechanical systems. In this work, alkaline treatment exhibits satis-

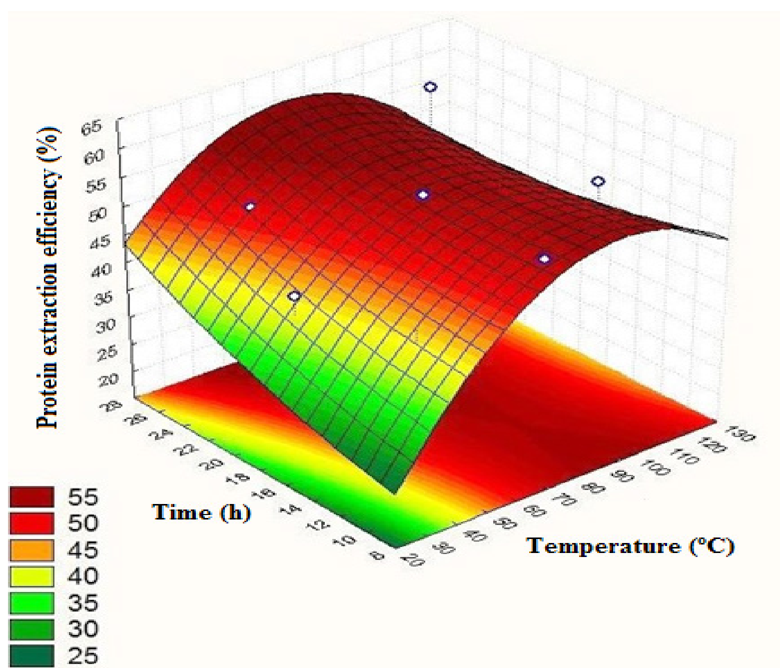

Figure 5. Response surface of protein extraction efficiency using alkaline hydrolysis.

factory results for extracting proteins from wet biomass (70.51 % of recovery).

The influence of thermal pre-treatment on the recovery of metabolites (proteins and carbohydrates) from *C.*

Figure 6. Pareto diagram for protein extraction using alkaline hydrolysis.

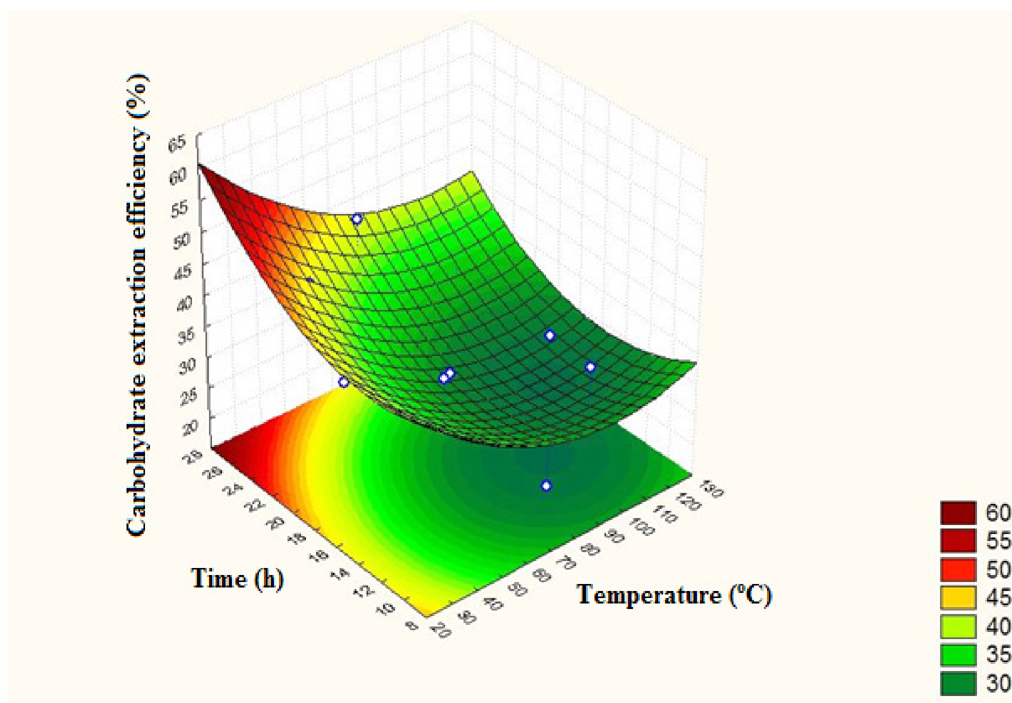

Figure 7. Response surface of carbohydrate extraction efficiency using alkaline hydrolysis.

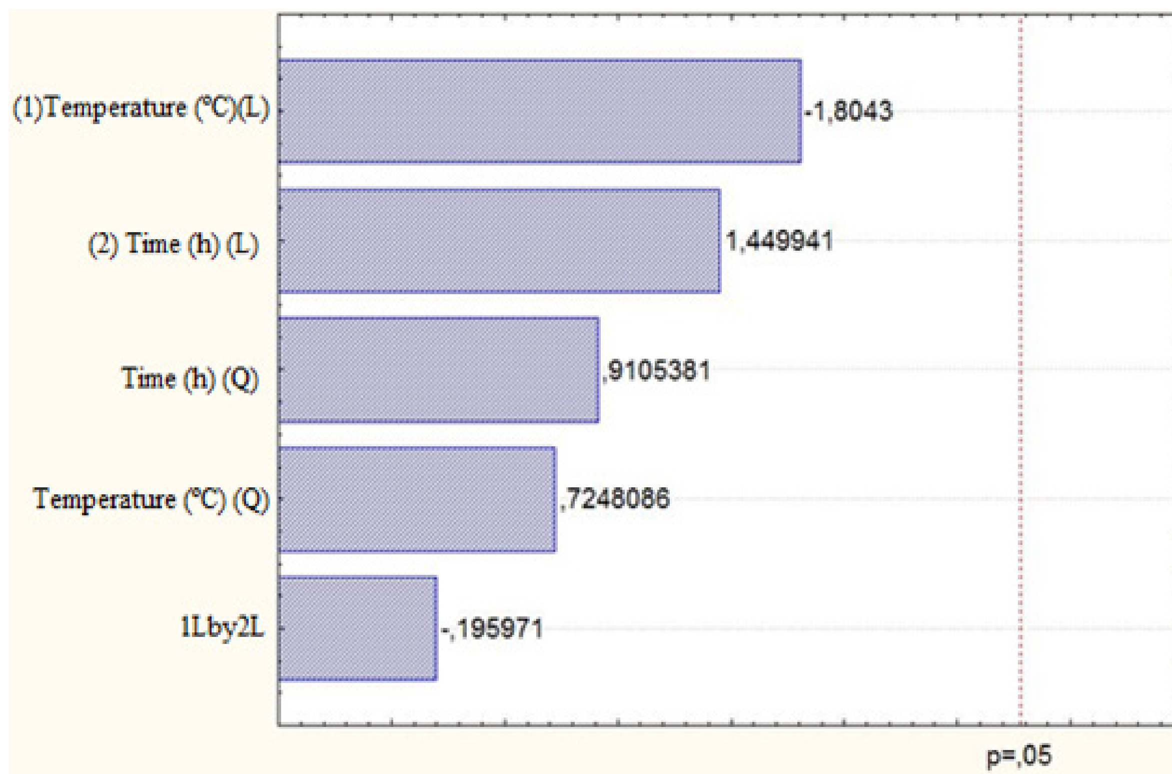

Figure 8. Pareto diagram for carbohydrate extraction using alkaline hydrolysis.

vulgaris, was analyzed by Statistica 7.0 software. Figure 5 shows the response surface of proteins extraction with temperature and time. It is observed that protein recovery achieves 55% when drying process is carried out at temperatures between 80 °C and 100 °C at any period of time. According to data performance, temperature during thermal pre-treatment affects the yield of protein recovery from *C. vulgaris*. Brennan & Owende[20] claim that the quality of metabolites can be affected by the increase in temperature during biomass drying.

Figure 6 shows that temperature and time during thermal pre-treatment did not significantly influence the extraction of proteins by alkaline treatment (NaOH). According to statistical analysis, it is inferred that producing proteins from *C. vulgaris* is not highly dependent on biomass drying conditions.

Figure 7 displays response surface of carbohydrate extraction with temperature and time. It is observed that extraction efficiency improves when biomass is exposed to ambient temperature (20 °C - 30 °C) during more than 24 hours. To verify this behavior, Pareto diagram was performed (Figure 8), where the effects or interactions that exceed the value P = 0.05 will be considered significant. According to statistical results, time and temperature variables of thermal pre-treatment have no influence on recovery of carbohydrates. Therefore, it is proposed to use hydrolysed wet biomass under alkaline conditions as an alternative to reduce costs, without significantly affecting the recovery of this metabolite.

3.3 Lipid Extraction using Hexane Method

The results of lipid extraction from *C. vulgaris* (dry and wet) using methodology proposed by Sathis & Sims[24] are represented in Figure 9. The highest lipid recovery (18.22 %) was achieved at 290.33 mg of biomass, 5 ml of hexane and 42.5 °C. It is observed that efficiency was not greater than 20% in any experiment. Sathis & Sims[24] reported recovery of 79% from mixed cultures of *Chlorella* and

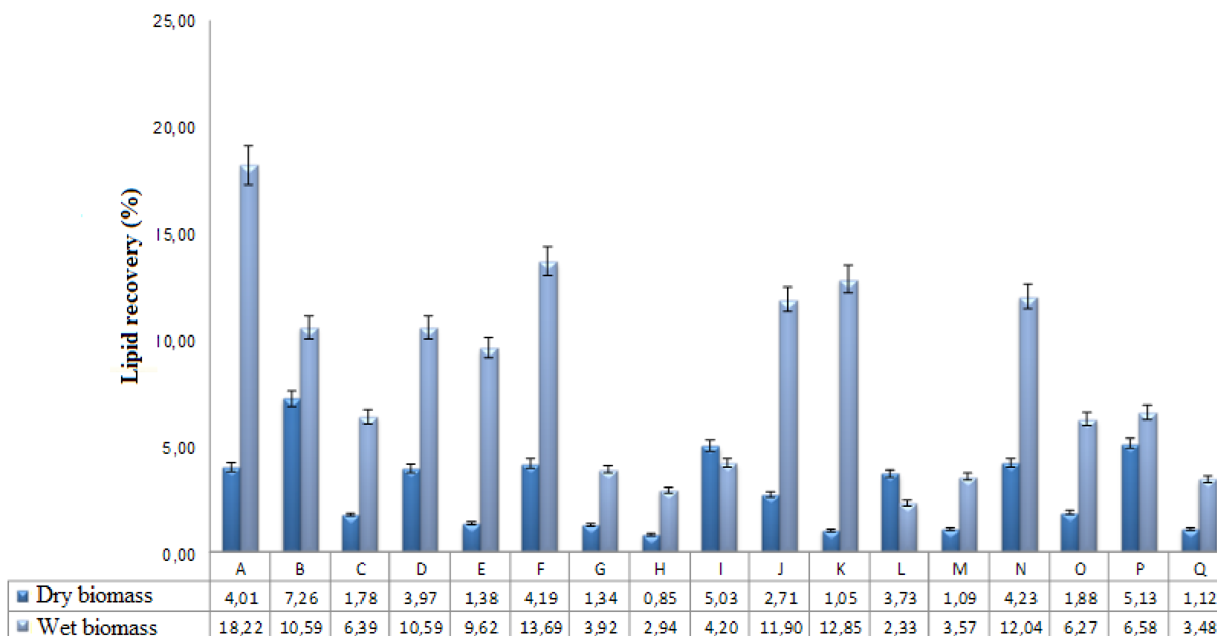

Figure 9. Lipid extraction using hexane method.

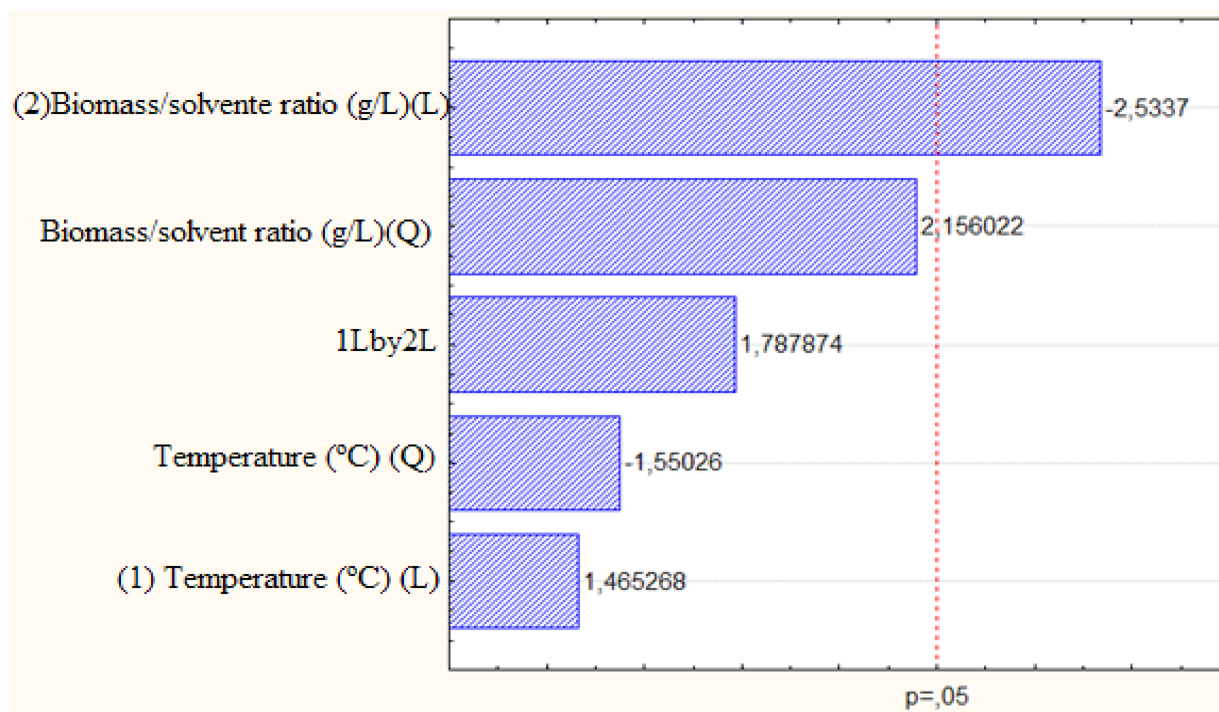

Figure 10. Pareto diagram for lipid extraction using hexane method.

Scenedesmus sp, using biomass with an average moisture of 84%. Other studies claim to be able to double the efficiency of lipid extraction from *Chlorella salina*, using alternative solvents to hexane such as 2-ethoxy ethanol (2-EE)[25].

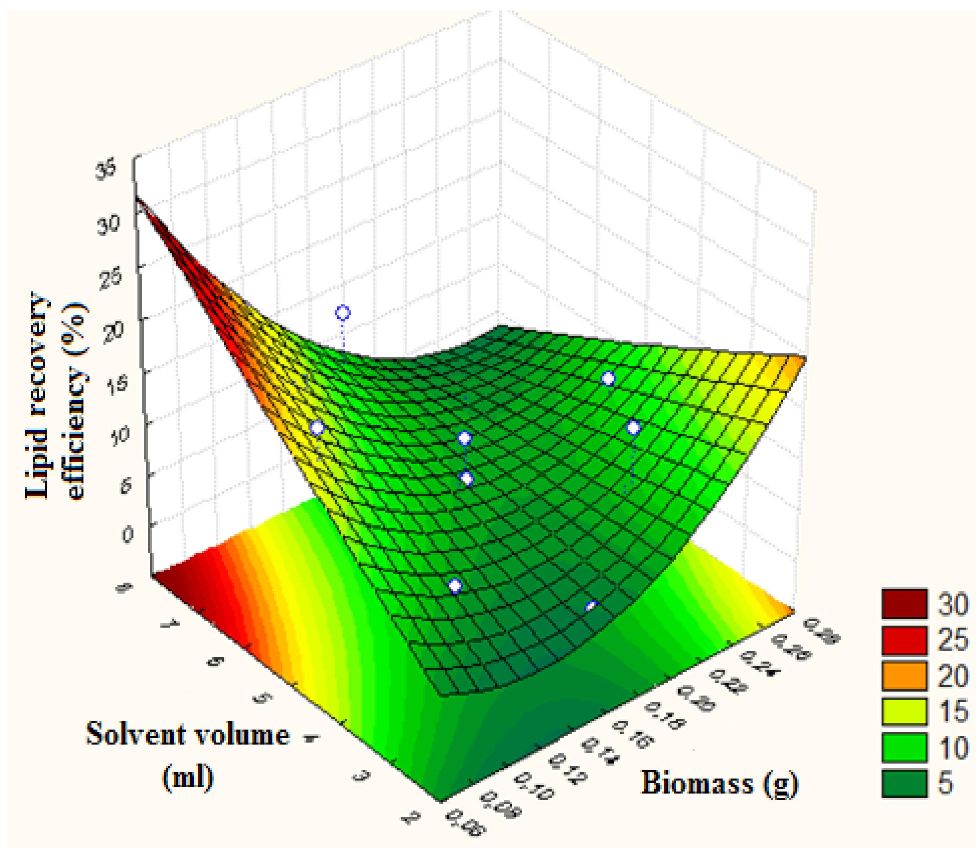

Figure 11. Response surface of lipid extraction efficiency using hexane as solvent.

The statistical analysis results are shown in Figure 10, which indicated that biomass/solvent ratio (g/L) was the variable with main influence on extraction efficiency from wet *Chlorella vulgaris*, using hexane method. On the other hand, temperature did not show a significant influence on this efficiency. However, some authors reported that greater amount of lipids are recovered from microalgae biomass (*Scenedesmus acutus*) at temperatures above solvent boiling point and high pressure conditions[26].

Figure 11 shows response surface of lipid extraction efficiency with volume of solvent and biomass amount. According to these results, extraction of lipids from wet *C. vulgaris* using hexane as solvent is directly influenced by volume of solvent, hence, efficiency of recovery increases when biomass/solvent ratio decreases.

3.4 Lipid Extraction using Methanol-Chloroform Method

The results for lipid extraction from *C. vulgaris* (dry and wet) using the methodology proposed by Bligh & Dyer[27] are represented in Figure 12. It was found that recovery of lipids exhibited values higher than 30% using methanol-chloroform solvent inmost of experiments. It is important to highlight that under biomass/solvent ratio of 5 g/L, 106 ml of methanol, 851 ml of chloroform and 43 ml of water, 97% of lipid was recovered. Some studies compare the efficiency of this method with other solvents. In[21] reported an efficiency of 97% in recovery of lipids from mixed cultures of 5 species of microalgae (moisture content of 85%) using di-methyl ether.

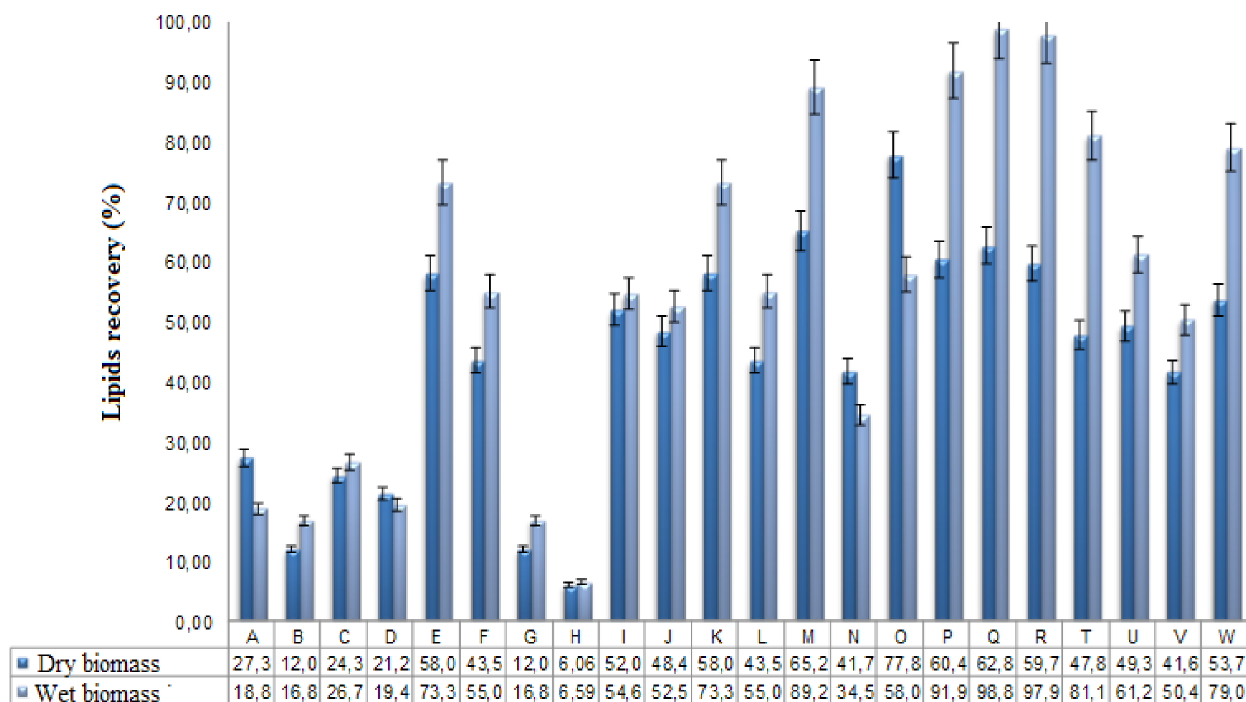

Figure 12. Lipid extraction using methanol-chloroform method.

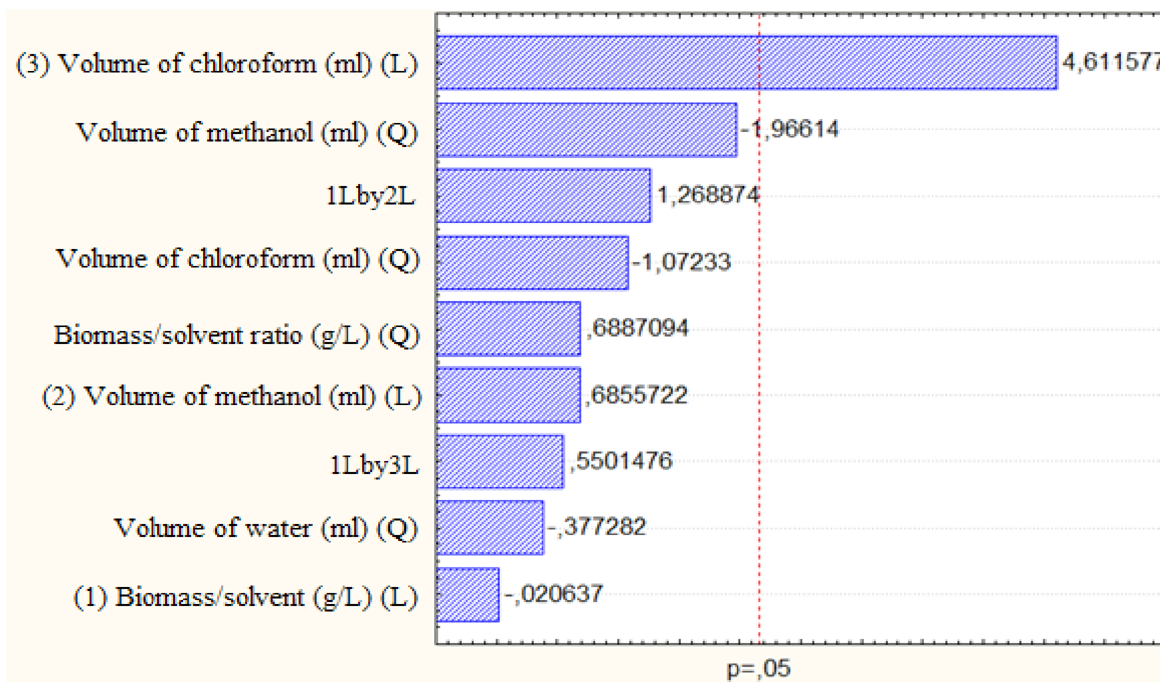

Figure 13. Pareto diagram for lipid extraction using methanol-chloroform method.

The statistical analysis shown in Figure 13 indicated that volume of chloroform (ml) was the variable with greatest influence on the recovery of lipids from wet *Chlorella vulgaris*, using the methodology proposed by Bligh & Dyer[27]. An increase in volume of chloroform enhances the recovery yield of lipid. In[28] reported

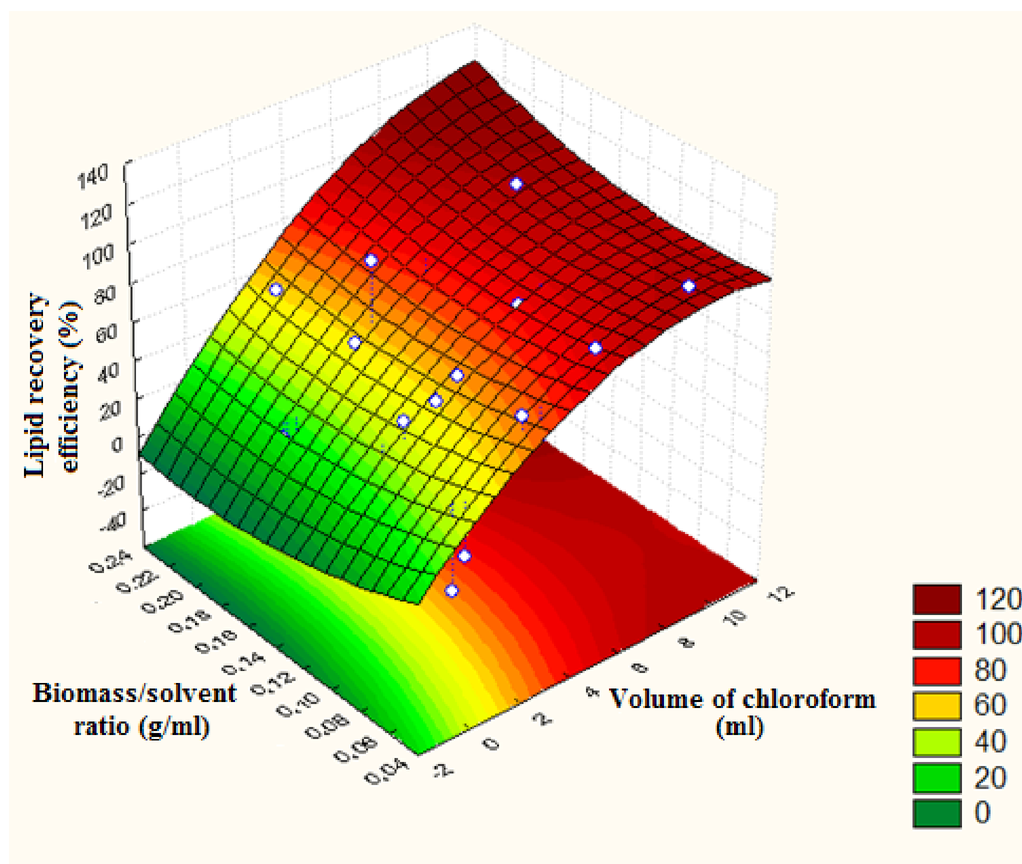

Figure 14. Response surface of lipid extraction efficiency using methanol-chloroform as solvent.

increases in efficiency of the process by varying concentration of chloroform. Figure 14 shows response surface of lipid extraction efficiency with volume of solvent and biomass content. It was observed this extraction is directly influenced by volume of chloroform in mixture ratio of solvent.

4. Conclusions

In this work, methods for extracting metabolites from microalgae *C. vulgaris* were studied in order to identify suitable conditions for carrying out carbohydrates, proteins and lipids extraction. It was found that variable temperature and time during thermal pretreatment did not affect significantly recovery yield, hence, biomass with 73% of moisture can produce 41.96% of carbohydrates using acid hydrolysis. In addition, recovery yield 71% of proteins y 49.77% of carbohydrates was obtained when alkaline hydrolysis was used. Lipid extraction reported recovery of 18.22 % and 97% using hexane and methanol-chloroform as solvents, respectively.

5. Acknowledgments

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