



Properties of microalgal enzymatic protein hydrolysates: Biochemical composition, protein distribution and FTIR characteristics[☆]



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ABSTRACT

Chlorella vulgaris SAG 211-12, a green microalga, as model organism was cultivated photomixotrophically using various organic carbon and nitrogen sources at Erlenmeyer scale. The modified medium selected for the experiments was standard BG11 supplemented with 5 g l⁻¹ glucose and 1 g l⁻¹ proteose peptone (PP). To evaluate the effects of light/dark cycles, 12:12; 18:6 and 24:0 light/dark cycle conditions were examined on hourly basis. 24:0 continuous illumination condition was chosen to continue 2 l continuous stirred tank photobioreactor (CSTR) experiments under 1 vvm aeration, 120 rpm mixing time, 23 ± 2 °C, and 70 μE m⁻² s⁻¹ illumination conditions. The results showed significant effect of the culture conditions on the cellular composition. To enhance digestibility of the intact cell; dry biomass was digested with pancreatin enzyme solution and *in vitro* protein digestibility (IVPD) of crude biomass (UTS), cell debris (CVA) and protein hydrolysates (CVH) was measured. IVPD values of UTS, CVA and CVH were found to be 33–41%, 46–58%, 67–89%; respectively with no significant changes regarding culture conditions (*p* > 0.05). Results also showed the positive effect of the enzyme treatment for digestion which is a key advantage for nutritional characteristic of the algal biomass.

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1. Introduction

Green microalgae are unicellular, eukaryotic, photosynthetic microorganisms living in fresh, saline or brackish water environments. They are composed of proteins, essential amino acids, fatty acids, antioxidant pigments, vitamins and other bioactive compounds that express unique features for the development of pharmaceuticals, nutraceuticals, cosmetics and biofuel industry [33,30]. The microalgal biotechnology showed a great progress in early 1950s with developments in processing technologies regarding the industrial applications of certain metabolites. The environmental conditions and nutritional modes alter the cell composition which can be used to produce diverse metabolites for various industries. The ease in cultivation, economically reliable features, high biomass efficiency and diversity of microalgal strains make the microalgae research even more attractive.

Among commercially important strains, *Chlorella vulgaris* has gained importance in terms of higher growth rates, high light to biomass conversion, ability to grow under phototrophic,

photomixotrophic and heterotrophic conditions, high protein amount, essential amino acids and fatty acids [34]. Besides *C. vulgaris* has been accepted as a functional food source with anticancer, immunoregulator, immunostimulating, antioxidant, antimicrobial activities [23,26,12,35].

C. vulgaris cells are surrounded by thick cellulosic wall composed of mostly hemicellulose fibrils and saccharides such as mannose, ramnose, xylose, galactose and glucose (Safi et al., 2014). Because of the thick cellulosic wall, the intact cell is poor in terms of digestibility [23]. There are several approaches to break down the cell integrity. However conventional methods such as acid or alkali hydrolysis damage the structure of free amino acids also low hydrolysis yield is another issue [15]. In that case; enzymatic digestion gives an opportunity to increase the yield of hydrolysis with increasing digestibility [23,24,4]. Enzymes like pepsin, papain, pancreatin, and trypsin are widely used to obtain enzymatic protein hydrolysates from conventional sources [24,31,10] other than bacterial proteases [3,2] that offer a mild process conditions and ease in the operation.

Protein hydrolysates are commonly used as food or drink additives to supplement protein value but another important aspect is their possible utilization for individuals who suffer from digestibility problems such as gastrointestinal malfunction or cystic fibrosis [5]. There are various studies in the literature covering different aspects of enzymatic protein hydrolysates [27,28] from conventional protein sources such as soybean or

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wey proteins [18,13]. Today considering the need of alternative sustainable food sources for the growing demand of global population microalgal hydrolysates are also emerging as attractive functional protein nutrition products.

The aim of this study is to cultivate *C. vulgaris* in photo-mixotrophic conditions and also see the effects of light/dark cycles to biochemical composition. As downstream process, the enzymatic hydrolysis is another key point of the study in order to enhance digestibility and gain a perspective in the field of microalgal dietary supplements. This can increase the preference of algal dietary supplements because when algal cells are disrupted the digestibility properties are enhanced besides the prevention of unwanted properties such as taste, fishy smell and green color can be avoided.

2. Materials and methods

2.1. Microalgae culturing and downstream processes

In this study; axenic cultures of *C. vulgaris* SAG 211-12 were used. Stock cultures of *C. vulgaris* were cultivated under continuous illumination of $40 \mu\text{E m}^{-2} \text{s}^{-1}$ in standard BG11 medium for 4–5 days. Cultures in mid-logarithmic phase were used as inoculum for photomixotrophic cultivation, light/dark cycles and continuous stirred tank photobioreactor (CSTR) experiments.

For photomixotrophic experiments; *C. vulgaris* cells were cultivated under continuous illumination of $70 \mu\text{E m}^{-2} \text{s}^{-1}$, $23 \pm 2^\circ\text{C}$, and 120 rpm agitation in 100 ml Erlenmeyer flasks. BG11 culture medium was modified with organic carbon sources (glucose (G), sucrose (S), fructose (F), glycerol or xylose); and organic nitrogen sources (yeast extract (YE), proteose peptone (PP) or urea) in various concentrations of 1 g l^{-1} and 5 g l^{-1} , respectively. Cultures were maintained for 7 days and sampled daily basis.

For light/dark cycle experiments, selected organic carbon and nitrogen sources were added to BG11. *C. vulgaris* cells were cultivated photomixotrophically under 12:12, 18:6 and 24:0 h light/dark cycles in 1 l of Roux-type flat photobioreactors with 800 ml of working volume. Cultures were maintained for 7 days and sampled on daily basis.

21 CSTR (Sartorius Biostat A-Plus, Germany) was used for bulk biomass cultivation under selected illumination strategy, at $23 \pm 2^\circ\text{C}$, and 120 rpm agitation conditions. Cells were centrifuged at $3000 \times g$ for 8 min for harvesting and washed twice with distilled water. Harvested cells were freeze dried and kept at -20°C until enzymatic hydrolysis.

2.2. Enzymatic hydrolysis

The enzymatic hydrolysis of crude microalgal biomass was done with pancreatin enzyme (Sigma–Aldrich, USA). The proteolytic activity of the enzyme was determined according to one

Table 1
The dry biomass and protein values of photomixotrophic production.

Component	Concentration (g l^{-1})	Dry biomass (g l^{-1})	Protein (mg g^{-1})
Glucose	1	1.58 ± 0.06	173.03 ± 9.84
	5	2.06 ± 0.08	176.08 ± 9.85
Sucrose	1	0.72 ± 0.03	184.42 ± 9.3
	5	0.73 ± 0.03	160.25 ± 10.28
Fructose	1	0.4 ± 0.02	152.61 ± 10.86
	5	1.35 ± 0.05	204 ± 8.26
Proteose peptone	1	0.92 ± 0.04	224.42 ± 8.22
	5	1.0 ± 0.04	218.31 ± 8.38
Yeast extract	1	0.6 ± 0.02	203.58 ± 8.94
	5	0.5 ± 0.02	210.53 ± 7.91

proteolytic unit expressed as the amount of enzyme necessary to catalyze the release of 1mmol tyrosine from 6% denatured casein solution at 37°C within 1 min (pH, 7.5).

1 g of dried *C. vulgaris* was mixed with 2.5% v/w distilled water (pH, 7.5) and incubated at 45°C for 1 h in temperature controlled shaker. The pancreatic hydrolysis was done for 4 h at 45°C with gentle shaking under selected E/S condition. The enzymatic reaction was stopped with heat treatment at 85°C for 20 min [23]. The hydrolyzed mixtures were centrifuged at $3000 \times g$ for 10 min. Supernatant (CVH) and cell debris (CVA) was freeze dried and kept at -20°C for further analysis.

2.3. Analytical methods

For dry biomass analysis; 2 ml of samples were filtered and washed with distilled water twice. Total dry biomass (g l^{-1}) was determined gravimetrically. Total soluble protein content was measured according to Lowry method [19]. Total gravimetric fatty acid was measured according to Bligh and Dyer method with some minor modifications [1].

In vitro protein digestibility (IVPD) was done according to Hsu multi-enzyme solution with some differences [9]. 5 mg of dried *C. vulgaris* biomass was mixed with 5 ml of distilled water (pH, 8). Trypsin (1.6 mg ml^{-1}) and α -chymotrypsin (1.6 mg ml^{-1}) enzyme mixture was dissolved in 1 ml distilled water (pH, 8). The pH drop is recorded for 10 min at 37°C . Sodium caseinate was used as reference sample with 100% IVPD value.

Sephadex G100 and G25 columns were used to determine peptide fractions of the samples. 5 g of Sephadex G100 and Sephadex G25 was weighted and kept in phosphate buffer (pH, 6.8) for 5 h. Columns were filled with Sephadex G100 ($2.5 \text{ cm} \times 25 \text{ cm}$) and G25 ($2.5 \text{ cm} \times 15 \text{ cm}$) and waited for 24 h. 2 mg of CVH was dissolved in 1 ml phosphate buffer (pH, 6.8) and 200 μl sample was injected to column. Eluted volume was collected and absorbance was measured at 214 nm in UV-visible spectrophotometer (Optizen Pop Korea). Bovine serum albumin (67 kDa), peroxidase (44 kDa) were used for G100 column; aprotonine (6.5 kDa) and cyanocobalamin (1.4 kDa) for G25 column were used as reference proteins.

Biochemical composition of dried UTS, CVH and CVA were characterized via FTIR spectroscopy (Perkin Elmer, USA). The samples were analyzed in between 4000 and 400 cm^{-1} wavelength and 2 cm^{-1} resolution with 4 parallel measurements.

2.4. Statistical analysis

The data analyzed by statistical tests using Microsoft Excel software. Statistical analysis consisted of summary statistics, including means, standard deviation and standard errors, where

Table 2
Total protein and fatty acid values of UTS, CVH and CVA.

	Condition	Fatty Acid (%)	Protein (mg g^{-1})
UTS	12:12	10.38 ± 0.32	394.76 ± 6.10
	18:6	11.7 ± 1.65	425.27 ± 4.2
	24:0	15.28 ± 1.52	499.8 ± 6.86
	CSTR	19.55 ± 2.6	545.47 ± 12.64
CVH	12:12	ND	226.48 ± 4.31
	18:6	ND	351.54 ± 4.5
	24:0	ND	447.09 ± 11.02
	CSTR	ND	443.85 ± 19.5
CVA	12:12	2.56 ± 0.03	168.3 ± 6.15
	18:6	3.045 ± 0.21	73.74 ± 5.2
	24:0	2.83 ± 0.17	52.72 ± 5.24
	CSTR	2.44 ± 0.05	101.61 ± 2.8

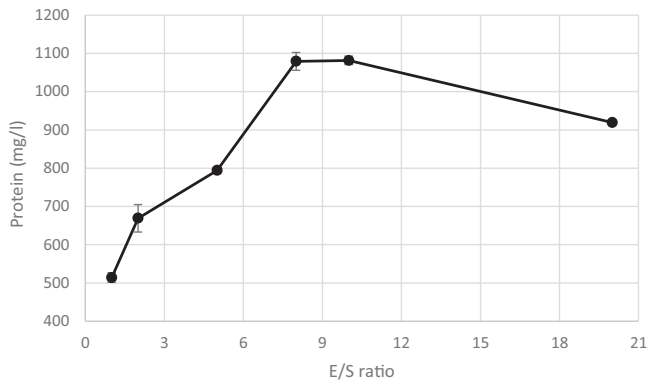


Fig. 1. The initial enzyme concentration.

one way analysis of variance was done, and then comparison was conducted at the 0.05 level. All the experiments were done with 3 replicates.

3. Results and discussion

Microalgal biotechnology is an emerging area with several aspects. Even though it is not a new technology the developing strategies, study topics and perspectives are spreading very fast enabling new understandings. In this study, the effects of nutrients and environmental conditions were investigated in order to obtain enzymatic hydrolysates as dietary supplements with increased digestibility.

3.1. Photomixotrophic cultivation experiments

Microalgae are capable of using several organic carbon and nitrogen sources for biomass production. Under control conditions (BG11 medium, phototrophic production) the total dry biomass was $0.74 \pm 0.03 \text{ g l}^{-1}$. Cultures supplemented with xylose were died in the early period of growth, some literature data also support these findings [14] meanwhile in glycerol, cells were able to reproduce but in 5 g l^{-1} experiments the growth was inhibited. Cell survival and production could be seen in cultures supplemented with glucose, sucrose and fructose (Table 1). The selected carbon source for *C. vulgaris* cells was glucose which gave highest biomass

value at 5 g l^{-1} experiment ($2.06 \pm 0.08 \text{ g l}^{-1}$). Other carbon sources also increased biomass accumulation however compared to glucose the growth was lower. Glucose is known to be a suitable organic carbon source for microalgal growth both for photo-mixotrophic and heterotrophic cultivation because it can diffuse through the cell very fast and it has more ATP potential for the cell growth [32].

The results showed that carbon sources did not have a significant effect on protein synthesis (Table 1). Some studies also suggest that carbon backbone of the organic carbon sources in culture media is used as precursors in starch or fatty acid metabolism in microalgae. Additional carbon input results as a dramatic increase in the biomass accumulation until the saturation point however does not stimulate protein accumulation.

In the case of organic nitrogen source experiments urea inhibited cell growth and increase in the urea concentration triggered cell death. But when PP and YE were used increase in the total protein was observed (Table 1) but did not significantly affect biomass accumulation. It is known that nitrogen sources are used for protein synthesis but increase in the concentration slightly decreased the protein amount, the reason is thought to be nitrogen accumulation in the culture media [32]. Other than the slight enhancement in the protein amount with PP compared to YE, the cultures grown in YE was contaminated in the later period of the growth lead the selection of the PP for the following experiments.

According to results; BG11 was supplemented with 5 g l^{-1} glucose and 1 g l^{-1} PP for light/dark cycle and CSTR experiments.

3.2. Light/dark cycles and CSTR experiments

Light is one of the major parameters affecting the regulation of microalgal metabolism. Some microalgae species can also grow in dark conditions but most of them are strict phototrophs. In this study, effect of light/dark cycles on hourly basis was investigated in photomixotrophic cultures.

The results briefly showed that, when illumination period was increased; the biomass accumulation is also increased. Biomass values of cultures were 1.76 ± 0.57 , 2.56 ± 0.38 ; $2.75 \pm 0.34 \text{ g l}^{-1}$, respectively. It can be seen that illumination enhances biomass production. In dark period cells only use respiration metabolism and storage materials such as starch, lipid or protein as nutrients which results in the weight losses of overall biomass amount known as night loss in naturally illuminated cultures [37].

Light/dark cycles also affect the biochemical composition of the cells [11]. As it can be seen from Table 2, with the increase in the illumination period also fatty acid and protein content is increased,

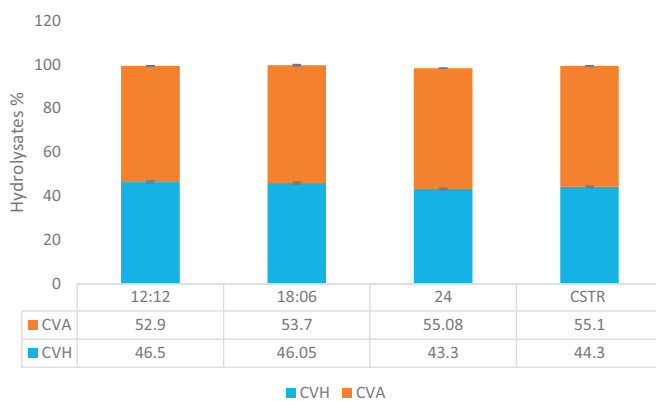


Fig. 2. Enzymatic hydrolysis of intact microalgae cells.

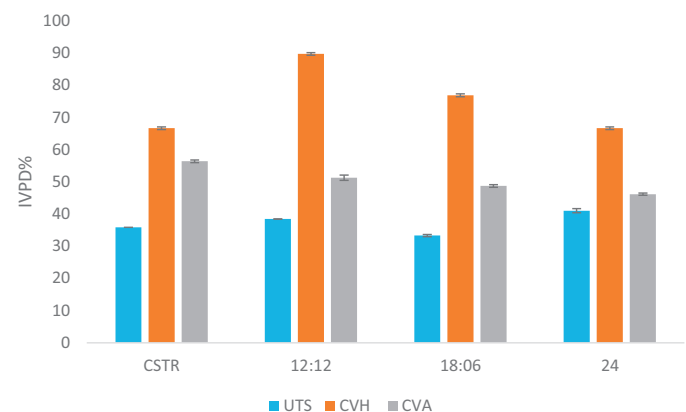


Fig. 3. IVPD% values of UTS, CVH and CVA.

a GRAS solvent, treatment also helped the cells to break down and increased the yield. The thick cellulosic walls of the cells are the main challenge in front of the enzymatic hydrolysis, which some of the studies also suggests pretreatments [4,28,29]. The choice of the enzyme is another issue to be emphasized. Pancreatin as a gastrointestinal enzyme is a dietary supplement for patients suffering from digestion problems. In that case aiming the enhancement of digestibility, pancreatin is the most widely used enzyme both for algal or conventional protein products [13,29,17].

The physical properties of CVH were yellow–white powder with no algal smell which is thought to be a good point for consumer's choice. Besides CVH, CVA is also an economically important compound that can be used in the formulation of dietary supplements (Table 2). The enzymatic hydrolysis broke down the cellulosic wall structure; the main barrier in front of the digestibility, besides it still had proteins, carbohydrates, lipids and cellulosic material.

3.4. IVPD values

IVPD values are one of the major properties of dietary supplements. Microalgal nutritional supplements suffer from low digestibility even they are consisted of valuable products [20]. In this regard IVPD values can give an idea about digestibility value and criteria of a compound to be used as a dietary supplement which this study aims to improve.

The specific activity of enzymes used for multi-enzyme solution was found to be 98 U/mg and 115 U/mg for trypsin and α -chymotrypsin, respectively. The IVPD values differed according to the state of the sample dramatically. UTS had IVPD value around 30–40%; meanwhile CVH had 67–89% IVPD value. Also CVA had increased IVPD values compared to UTS (Fig. 3). The IVPD values were not significant according to culturing conditions but had a significant impact in between UTS, CVH and CVA [36]. According to results obtained from IVPD studies, CVH is a suitable protein supplement with high protein content and enhanced digestibility.

3.5. Protein fractioning

Molecular weight distribution of CVH was determined *via* gel filtration columns. According to the molecular weight analysis CVH phase was divided into 2 main classes; CVH-100 representing peptides fractioned with Sephadex G100 column and CVH-25 fractioned with Sephadex G25 column. Molecular weight distribution of CVH-100 for 12:12, 18:6 and 24:0 were in a broad range. These peptides were also divided into 3 major classes representing large peptides (>50 kDa), medium peptides (in between 30 and 50 kDa) and small peptides (<30 kDa). Peptide with 125 kDa molecular weight was observed in light/dark cycle experiments. In CSTR culture peptide distribution started with medium peptides. In CVH-25 molecular weights of the peptides were diverse with no significant common peptide (Table 3).

The gel filtration column gave an idea about molecular weight patterns of the hydrolysates. The reason to have a broad spectrum of peptides is thought to be non-specific action of the pancreatin enzyme [21] because of the globular structure of the algal proteins within the cells was a limiting step for smaller fractioning [29].

3.6. FTIR analysis

FTIR is a new and efficient technique which is helpful to analyze the biochemical composition of microalgal cells in a short period of time without complex sample preparation [25]. In this study FTIR is used to determine the biochemical composition of UTS (Fig. 4), CVH (Fig. 5) and CVA (Fig. 6). The UTS for light/dark cycle and CSTR experiments gave the same wavenumbers with no significant difference but it was seen that the peak areas were differed according to cultivation conditions of the cells. It was found that UTS was composed of proteins (3280 cm^{-1} , 1642 cm^{-1} amid I, 1542 cm^{-1} amid II, 1398 cm^{-1} carboxylic group, lipids (2923 cm^{-1}), carbohydrates (1040 cm^{-1}), cellulose ($1165\text{--}1000\text{ cm}^{-1}$) and nucleic acids (1241 cm^{-1}). In CVH samples the major bands were $3162\text{--}3187\text{ cm}^{-1}$, 2928 cm^{-1} , $1577\text{--}1583\text{ cm}^{-1}$, 1399--

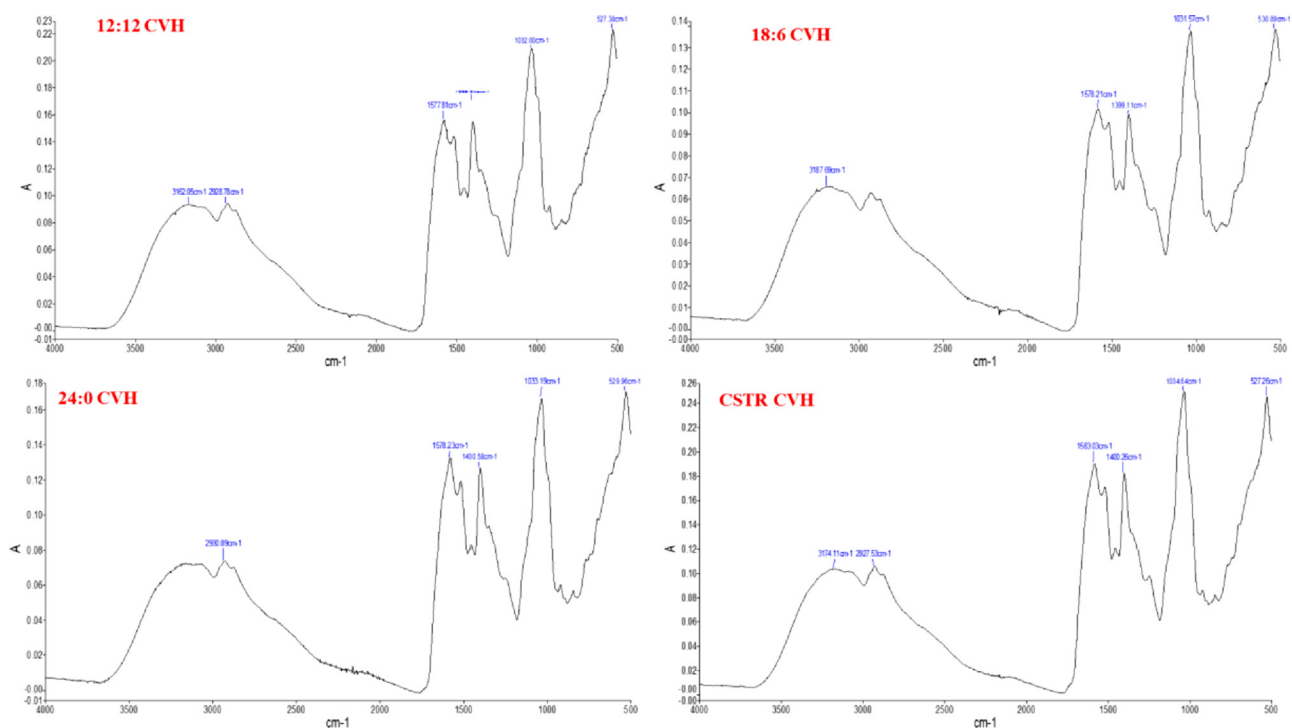


Fig. 5. FTIR analysis of CVH.

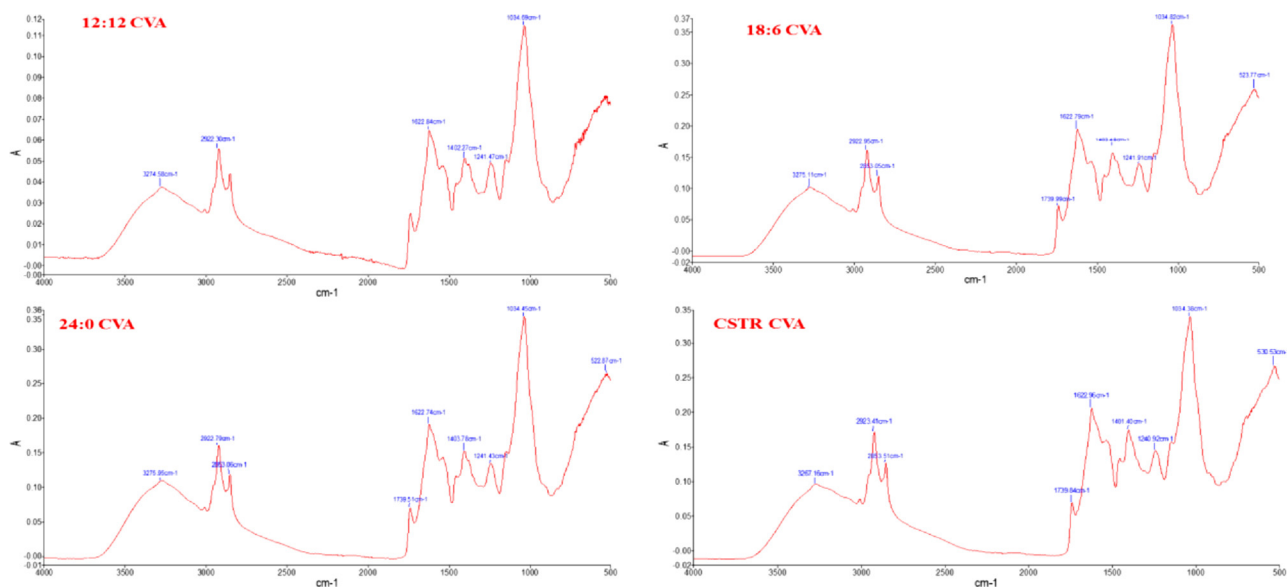


Fig. 6. FTIR analysis of CVA.

1402 cm^{-1} , 1031–1034 cm^{-1} and 527–529 cm^{-1} . For CVA samples the FTIR bands are resulted as 3274 cm^{-1} , 2922 cm^{-1} , 1622 cm^{-1} , 1402 cm^{-1} , 1241 cm^{-1} , 1034 cm^{-1} and 522 cm^{-1} .

The FTIR spectra has shown a very important result in terms of the efficiency of hydrolysis that the lipids, proteins, carbohydrates and cellulose bands were also found in CVA phase. The most important result obtained from FTIR analysis was the absence of the nucleic acids in CVH phase [29]. CVH was consisted of mainly proteins and carbohydrates. Also there were no relevant bands representing cellulose, a criteria for digestibility value.

4. Conclusion

Changes in the scientific perspectives also opened new insights in microalgal biotechnology leading researches related to innovative substances. In this study, aim was to see the effect of environmental conditions and nutritional mode on the biochemical composition of microalgae cells in the scope of protein synthesis. Also the emphasis was to increase the digestibility of intact algae cells in order to use for patients suffering from digestion problems especially patients with cystic fibrosis. A strong point for this study is the use of FTIR spectra as a tool to determine hydrolysates composition. Other than the importance of cystic fibrosis the relation of microalgal protein hydrolysates regarding the supplemental nutrition for cystic fibrosis patients were also wanted to be stressed to catch more attention for the possible improvement chances of the life quality of the patients. The results are promising in terms of increased digestibility and biochemical composition of CVH is a good motivation for the future studies focusing on cystic fibrosis cell lines [16].

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