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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.

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 pharmaceutials, 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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whey 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 photomixotrophic 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 $\mu E\,m^{-2}\,s^{-1}$ 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 $\mu E\,m^{-2}\,s^{-1}$, 23 ± 2 °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: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.

2 l CSTR (Sartorious Biostat A-Plus, Germany) was used for bulk biomass cultivation under selected illumination strategy, at 23 ± 2 °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 proteolitic activity of the enzyme was determined according to one proteolytic unit expressed as the amount of enzyme necessary to catalyze the release of 1nmol 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 cm × 25 cm) and G25 (2,5 cm × 15 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; approtinine (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 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

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