Whole-Cell Protein Profiles of Disintegrated Freshwater Green Algae and Cyanobacterium

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* Corresponding author: Ladislava Mišurcová; Tel.: +420 576031201; fax: +420 577210172; e-mail address: misurcova@ft.utb.cz

Dušan Samek[1], Ladislava Mišurcová[2,*], Ludmila Machů[1], Leona Buňková[3], Antonín Minařík[4], Miroslav Fišera[1]

[1]Tomas Bata University in Zlín, Department of Food Analysis and Chemistry, nam. T.G.Masaryka 5555, 760 01 Zlín, Czech Republic

[2]Tomas Bata University in Zlín, Department of Food Technology, nam. T.G.Masaryka 5555, 760 01 Zlín, Czech Republic

[3]Tomas Bata University in Zlín, Department of Environmental Protection Engineering, nam. T.G.Masaryka 5555, 760 01 Zlín, Czech Republic

[4]Tomas Bata University in Zlín, Department of Physics and Materials Engineering, nam. T.G.Masaryka 5555, 760 01 Zlín, Czech Republic

ABSTRACT. Influence of cultivation methods and post-harvesting treatment on protein profiles of green freshwater microalgae *Chlorella kessleri*, *Scenedesmus quadricauda*, and *Chlorella* sp. and cyanobacterium *Spirulina platensis* were evaluated. The comparison of protein profiles in algal biomass originated from the autotrophic cultivation in an outdoor open circulating cascade-type cultivation apparatus in thin-layer, a solar photobioreactor and from the heterotrophic cultivation regime in a fermenter. All tested algae contained protein bands in the area between 14.3 – 27 kDa and 70 – 116 kDa. Protein profiles revealed much higher heterogenity in the area between 30 – 70 kDa.

KEYWORDS. Protein profile, cultivation methods, disintegration, green freshwater microalgae, cyanobacterium

INTRODUCTION

Microalgae has been used by the human population for hundreds of years as food, feed, therapeutics, and fertilizers (Barsanti and Gualtieri, 2006). Microalgae contains a broad spectrum of nutritious compounds, including proteins (amino acids), carbohydrates (polysaccharides), lipids (fatty acids), vitamins (A, B_1, B_2, B_6, C, E), pigments (chlorophyll, carotenoids), and minerals. High protein and amino acid contents in algae may be considered as one of the relevant reasons for using these organisms as an unconventional source of nutrition (Becker, 2007; Morris et al., 2008; Spolaore et al., 2006).

Recently, there has been growing interest in using green freshwater microalgae from chlorophyceae, such as *Chlorella* and *Scenedesmus*, and cyanobacteria, such as *Spirulina*, as an alternative source of amino acids and proteins for human nutrition (Gouveia et al., 2007; Morris et al., 2008; Spolaore et al., 2006). Protein content in algal biomass depends on factors such as the geographic area, species of algae, environmental factors, and the method and conditions of cultivation (Barsanti and Gualtieri, 2006; Becker, 2007; Gouveia et al., 2007; Spolaore et al., 2006; Zhou et al., 2005). The presence of cellulose in green freshwater algae cell walls is the limiting factor for utilization of nutritional components in algal biomass by non-ruminants due to absence of enzyme cellulase (Becker, 2007). Various methods of post-harvesting treatment of

algal biomass should be developed for the utilization of nutritional factors present in algal biomass.

There have been some studies on the protein composition of algal biomass, but there is still a lack of information about protein profiles in algal biomass from various algal strains originating from the cultivations provided under different conditions. Further, the changes in algal protein profiles after the post-harvesting treatment of algal biomass by mechanical nethods have not been evaluated.

The aim of this research was the determination of protein profiles of selected green freshwater algae *Chlorella kessleri*, *Scenedesmus quadricauda*, and *Chlorella* sp. and blue-green alga (cyanobacterium) *Spirulina platensis* cultivated by various methods and the evaluation of the influence of the post-harvesting mechanical treatment on protein profiles of disintegrated algal biomass. Finally, the homology of protein profiles between investigated samples of algae was established.

MATERIALS AND METHODS

Reagents and chemicals. For the total protein analysis, Tris-buffer, EDTA, and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Protein Marker, Broad Range, 2-212 kDa, P7702L (New England Biolabs Inc., Ipswich, MA, USA), containing proteins of defined molecular mass (212, 158, 116, 97.2, 66.4, 55.6, 42.7, 34.6, 27 20, 14.3, 6.5, 3.4 and 2.3 kDa), was used as a molecular weight marker. All other chemical reagents were of analytical grade.

Algal materials. The series of species of green freshwater microalgae *Chlorella kessleri*, *Scenedesmus quadricauda* and blue-green alga *Spirulina platensis* were obtained from Academic and University Center of Nové Hrady, Institute of Physical Biology, Nové Hrady, Czech Republic; alga *Chlorella* sp. was obtained from Academy of Science of the Czech Republic, Institute of Microbiology, Department of Phototrophic Microorganisms in Třeboň.

The samples of algae (*C. kesslleri*, *S. quadricauda, S. platensis*) from Institute of Physical Biology in Nové Hrady were cultivated autotrophically in a solar photobioreactor (PBR). *Chlorella* sp. obtained from Department of Phototrophic Microorganisms in Třeboň was autotrophically cultivated in an outdoor open circulating cascade-type cultivation apparatus in thin-layer. Finally, the heterotrophic cultivation of *Chlorella* sp. in a fermenter was also performed. Summary and detailed description of the investigated samples is shown in Table 1. All investigated samples were acquired in dried forms.

Autotrophic cultivation in solar photobioreactor. The algal species *C. kesslleri*, *S. quadricauda,* and *S. platensis* were autotrophically cultivated in a solar PBR according to Masojídek et al. (2009). For the cultivation of algae, BG11 culture medium was used (Stanier et al., 1971). Cultivated algal biomass was lyophilized and used for analyses.

Autotrophic cultivation in outdoor open circulating cascade-type cultivation apparatus in thin-layer. Alga *Chlorella* sp. was autotrophically cultivated in an outdoor open circulating cascade-type cultivation apparatus in thin-layer as described in previous studies (Doucha and Lívanský, 2009; Masojídek et al., 2010). Briefly, cells were maintained in the medium with the composition as follows (per 1 L): 182 mg $(NH_2)_2CO$, 41 mg KH_2PO_4, 29 mg $MgSO_4.7H_2O$, 5.1

mg $FeSO_4.7H_2O$; trace element solution I (per 1 L): 141 µg H_3BO_3, 160 µg $CuSO_4.5H_2O$, 559 µg $MnCl_2.4H_2O$, 105 µg $CoSO_4.7H_2O$, 455 µg $ZnSO_4.5H_2O$; trace element solution II (per 1 L): 29.1 µg $(NH_4)_6Mo_7O_{24}$, 2.37 µg NH_4VO_3. The medium was sterilized by autoclaving. After the cultivation, the algal biomass was harvested, spray dried, and used for analyses.

Heterotrophic cultivation in fermenter. Chlorella sp. was heterotrophically cultivated in a fermenter (the volume of 450 L) at the temperature of 35-37 °C in the dark. Cells were maintained in the medium with the composition as follows (per 1 L): 77.8 g $C_6H_{12}O_6$, 7.11 g $(NH_2)_2CO$, 16.44 g KH_2PO_4, 1.22 g $MgSO_4.7H_2O$, 97.3 mg $FeSO_4.7H_2O$, 22.2 mg H_3BO_3, 6.22 mg $CuSO_4.5H_2O$, 8.66 mg $ZnSO_4.7H_2O$, 7.55 mg $CoSO_4.7H_2O$, 10.11 mg $MnCl_2.4H_2O$, 3.55 mg $(NH_4)_6Mo_7O_{24}.4\ H_2O$, 54 mg $CaCl_2$. The medium was sterilized by autoclaving. The cultivation medium in fermenter was stirred by blender and simultaneously was aered by using hypertensive air which was led through a flowmeter and microbial filter inside the fermenter. After the cultivation, the suspension of algal biomass was transferred into a washing tank where it was diluted by water, and further the suspension was concentrated in a disc centrifuge. This concentrated suspension of algal biomass was spray dried and used for analyses.

Basic chemical analyses of algae. Contents of crude protein, ash, and dry matter in the investigated samples were analyzed according to AOAC approved methods (AOAC, Official methods of analysis, 1995). Crude protein content was calculated using a conversion factor of 6.25.

Post-harvesting treatment of algal biomass. Two methods of post-harvesting treatment of algal biomass by mechanical methods were provided by using an oscillatory globe mill (MM 301,

Retsch, Germany) and by using glass microbeads Balotina B7 570–700 μm (Preciosa Ornela a.s., Czech Republic).

Treatment of algal biomass by oscillatory globe mill. Disintegration of algal biomass using an oscillatory globe mill was performed for 7 minutes at the frequency of 15 s^{-1} in a metal cell with a metal ball. The effectivity of the disintegration process was observed in a microscope (Nikon Eclipse 50i, Tokyo, Japan) in the immersion oil at maximum enlargement (Figure 1). Disintegrated algal material was used for analyses.

Treatment of algal biomass by glass microbeads Balotina B7. Disintegration of algal cell walls by glass microbeads Balotina B7 was performed in plastic tubes. Plastic tubes with the algal sample and Balotina B7 and distilled water were inserted into a modified metal cell of an oscillatory globe mill. Disintegrated process was held for 30 minutes at the frequency of 30 s^{-1}. Subsequently, glass microbeads were removed and algal biomass was immediately lyophilized (Alpha 1-4 LSC, Christ, Germany). Lyophilized samples were used for analyses. The degree of disintegration of algal biomass was observed in a microscope as it was mentioned above.

Whole-cell protein analysis. Sodium dodecyl sulfate vertical polyacrylamide gel electrophoresis (SDS-PAGE) (Mini-Protean-Tetra Cell, Power Pac Universal, BIO-RAD, Hercules, CA, USA) was used for the total protein analysis in algal biomass. The method was provided according to Leu and Hsu (2005). Each sample migration in SDS-PAGE was repeated three times. Gel was evaluated by using the program Bio.1D version: 12.11 (Vilber Lourmat, France); dendrograms were calculated by the UPGMA clustering method using Dice coefficients at a significance level of 5 %.

RESULTS AND DISCUSSION

Basic chemical analyses of algae. Results of dry matter, ash, and crude protein of investigated algae are shown in Table 2. Values of dry matter contents in investigated algae were steady and oscilated above 90 %; the highest value (96.0 %) was established in *S. quadricauda*. Autotrophically cultivated *C. kessleri* and *Chlorella* sp. were similar in content of dry matter and crude protein, but algal biomass of *C. kessleri* was richer in ash content. The highest ash content was determined in *S. platensis*; remaining algal samples were about three times lower in this parameter. Blue-green alga *S. platensis* was the most abundant source of crude protein; conversely, heterotrophically cultivated *Chlorella* sp. contained the lowest amount of crude protein. This situation is probably caused by dissimilar sensitivity of various algal strains to different conditions during the autotrophic and heterotrophic cultivation (the illumination intensity, chemical composition of substances in the culture medium, or temperature during the cultivation).

Protein profiles of algae and their homology. Whole-cell protein fingerprints of investigated algae are shown in Figure 2; further homology of the analyzed samples is illustrated in dendrogram (Figure 3). The distribution of protein molecular weight in algae was determined by SDS-PAGE electrophoresis. Protein molecular weights obtained from this method do not represent the protein molecular weights in the samples but only the molecular weights of protein subunits (Chronakis et al., 2000). Dissociation of protein complexes into subunits is caused by a presence of reducing agent (e.g. sodium dodecyl sulphate).

Higher number of bands in the area between 14.3 – 27 kDa and 70 – 116 kDa were observed in the profiles of all tested algae. Protein profiles revealed much higher heterogenity in the area between 30 – 70 kDa. A protein at 30 – 70 kDa was not expresed by *S. platensis*; additionally, *Chlorella* sp. and *C. kessleri* were absent of protein bands at approximately 66.4 to 75.0 kDa. Further, *S. quadricauda* did not display protein bands between 50 – 70 kDa. A certain degree of variability was evident in protein profiles of *Chlorella* sp. and *S. quadricauda.*

C. kessleri contained a high number of bands in the area between 10 – 30 kDa and 80 – 120 kDa in accordance with *Chlorella pyrenoidosa* from photoautotrophic laboratory cultivation according to Leu and Hsu (2005). On the other hand, significant differences of their protein profiles were observed in the area between 30 – 60 kDa, which was abundantly presented in *C. pyrenoidosa*, while bands from this area in *C. kessleri* were missed.

Protein profile of *S. platensis* presented in our work was partially similar to the profile of the same cyanobacterium shown by Chronakis et al. (2000) originating from open pond system cultivation. A certain dissimilarity between protein profiles was observed in the interval from approximately 43 to 67 kDa. The presence of proteins from this area was not confirmed in our study.

Relatively high homology of protein profiles was evaluated between investigated alga *S. quadricauda* cultivated in PBR and alga *Scenedesmus obliquus* described in the study by Osman et al. (2004), where it was cultivated autotrophically in Kuhl medium under laboratory conditions. However, comparison of protein profiles of *S. quadricauda* and *S. obliquus* indicated differences among their profiles, the heterogenity mainly revealed in the area between 43 – 69

kDa. *S. obliquus* contained proteins in this interval, conversely to *S. quadricauda*, without protein fractions of mentioned molecular weights.

Most of the proteins from algae are originated from the photosynthethic apparatus that are aggregated in super molecular structures. In blue-green algae, phycobiliproteins are constituent of macromolecular light-harvesting complex protein (LHC), and phycobilisome is associated with the thylakoid membranes in chloroplasts (Chronakis et al., 2000).

Protein profiles of investigated green freshwater and blue-green algae are mainly formed by polypeptides associated with photosynthesis. Protein bands from interval 4-36 kDa are predominantly formed by polypeptides originating from photosystems I and II. Polypeptides from thylakoid membranes, light-harvesting complex proteins (LHC IIc, LHCPa, LHCPb) and phytochelatins, and polypeptides of P700-chlorophyll *a*-protein 1 from photosystem I are displayed as protein bands 8, 10, 15 and 18 kDa; molecular weight 9 kDa could belong to protein CPIII - Chlorophyll *a* protein; and the area around 22 kDa may indicate protein CP22 (Brandt et al., 1982; Funk et al., 1994; Hoj and Moller, 1986; Ikeuchi and Inoue, 1988; Morishige and Thornber, 1994; Osman et al., 2004). Protein bands around 40 and 70 kDa may belong to the enzyme magnesium chelatase (termed I and D); moreover, bands 75 kDa are adherent to protein Toc75 (Gamini Kannangara and Wettstein, 2010; Inoue and Potter, 2004). Polypeptides from photosystem I complex (PSI-200) are displayed as protein bands 58 and 62 kDa (Malkin, 1986). The presence of bands around 100 and 110 kDa may be induced by chlorophyll a protein CPIV and P700-chlorophyll *a* protein 1 from photosystem I (Brandt et al., 1982; Hoj and Moller, 1986).

Dendrogram illustrated homology of the investigated algae is shown in Figure 3. The highest similarity of protein profiles was determined between algae *S. platensis* and *C. kessleri*; it was at the level of 69 %. Further similarity of protein profile *S. quadricauda* to previously mentioned algae was lower at the level of 57 %. The largest heterogenity of protein profile was noted in alga *Chlorella* sp.; its similarity was only 21 % to other algae. Further, the similarity of protein profiles of autotrophically and heterotrophically cultivated *Chlorella* sp. was determined; homology of their profiles was at the level of 81 %.

No effect of disintegration of algal biomass by oscillatory globe mill or glass microbeads Balotina B7 on protein profiles of any investigated algae were observed. It may be deduced that algal protein in biomass were stable and resistant to presented methods of the post-harvesting treatment by mechanical methods.

Data from presented work are predominatly in agreement with studies by Chronakis et al. (2000), Leu and Hsu (2005), and Osman et al. (2004), although there were some differences in protein profiles with compared algae. It may be concluded that the protein profiles of green freshwater algae and cyanobacterium varied in dependence on different species of algae; moreover, it may be different between algae originated from the same genus. Heterogenity between protein profiles of algae can be caused by the method of determination of protein profile. Further, different protein contents by virtue of different cultivation methods can also influence algal protein profiles.

CONCLUSION

Protein profiles in the selected cyanobacterium and green freshwater algae were predominantly formed by proteins in the area between 10 – 35 kDa and 95 - 116 kDa. However, it is evident that protein profiles of investigated algae are specific for each algal genus; even within the *Chlorella* genus, there were noted significant dissimilarities in protein profiles between *Chlorella* sp. and *C. kessleri.* It was observed and evaluated that cultivation method may directly induce changes in protein profiles of investigated algae.

Further, the pilot study investigated the effect of the post-harvesting treatment to algal biomass by mechanical method using an oscillatory globe mill and glass microbeads Balotina B7 for modification of algal protein profiles. The post-harvesting treatment of algal biomass by mechanic method did not influence the protein profile in algae. Described methods of the post-harvesting treatment of algal biomass may be applicable in further research as a suitable treatment method of algal biomass that does not affect protein composition.

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TABLE 1 Summary and description of investigated samples of blue-green and green freshwater algae.

Symbol of sample	Alga	Specification of alga		
		Cultivation	Culture medium	Disintegration method
SpAP	*Spirulina platensis*[a]	AP	BG11	---
SpAPM	*Spirulina platensis*[a]	AP	BG11	oscillatory globe mill (M)
SpAPB	*Spirulina platensis*[a]	AP	BG11	Balotina B7 (B)
ScAP	*Scenedesmus guadricauda*[a]	AP	BG11	---
ScAPM	*Scenedesmus guadricauda*[a]	AP	BG11	oscillatory globe mill (M)
ScAPB	*Scenedesmus guadricauda*[a]	AP	BG11	Balotina B7 (B)
ChKAP	*Chlorella kessleri*[a]	AP	BG11	---
ChKAPM	*Chlorella kessleri*[a]	AP	BG11	oscillatory globe mill (M)
ChKAPB	*Chlorella kessleri*[a]	AP	BG11	Balotina B7 (B)

ChAO	*Chlorella* sp.[b]	AO	for autotrophic cultivation[c]	---
ChAOM	*Chlorella* sp.[b]	AO	for autotrophic cultivation[c]	oscillatory globe mill (M)
ChAOB	*Chlorella* sp.[b]	AO	for autotrophic cultivation[c]	Balotina B7 (B)
ChHF	*Chlorella* sp.[b]	HF	for heterotrophic cultivation[c]	---

[a] Academic and University Center of Nove Hrady, Institute of Physical Biology, Nové Hrady, Czech Republic; autotrophic cultivation in solar photobioreactor (AP)

[b] Academy of Science of the Czech Republic, Institute of Microbiology, Department of phototrophic microorganisms, Třeboň, Czech Republic; autotrophic cultivation in outdoor open circulating cascade-type cultivation apparatus in thin-layer (AO), heterotrophic cultivation in fermenter (HF)

[c] Composition of cultivation medium described in method section

TABLE 2 Results of basic chemical analysis (crude protein content, ash content and dry matter content) in blue-green and green freshwater microalgae (Sp – *Spirulina platensis*; ChK – *Chlorella kessleri*; Sc – *Scenedesmus quadricauda*; Ch – *Chlorella* sp.) after the autotrophic cultivation in a solar photobioreactor (AP), the autotrophic cultivation in an outdoor open circulating cascade-type cultivation apparatus in thin-layer (AO) and the heterotrophic cultivation in a fermenter (HF); (%; N=3; mean values ± S.D.).

Sample	Alga	Cultivation method	Crude protein (%)	Ash (%)	Dry matter (%)
SpAP	*S. platensis*	AP	55.6 ± 0.6	17.8 ± 0.4	91.5 ± 0.2
ScAP	*S. quadricauda*	AP	43.4 ± 0.7	5.2 ± 0.1	96.0 ± 0.5
ChKAP	*C. kessleri*	AP	53.3 ± 0.7	6.7 ± 0.6	94.0 ± 0.1
ChAO	*Chlorella* sp.	AO	54.9 ± 0.4	4.8 ± 0.3	93.8 ± 0.4
ChHF	*Chlorella* sp.	HF	31.6 ± 0.9	4.8 ± 0.3	94.7 ± 0.8

Figure 1 Microscopic observation of investigated blue-green a green freshwater microalgae. The post-harvesting treatment of algal biomass was provided as described in section materials and methods. Sample of algae: 1 – Chlorella sp.; 2 – Chlorella kessleri; 3 – Scenedesmus quadricauda; 4 – Spirulina platensis. Treatment of samples: A – origin sample (non disintegrated); B – algal biomass treated by glass microbeads Balotina B7; C – algal biomass treated in oscillatory globe mill.

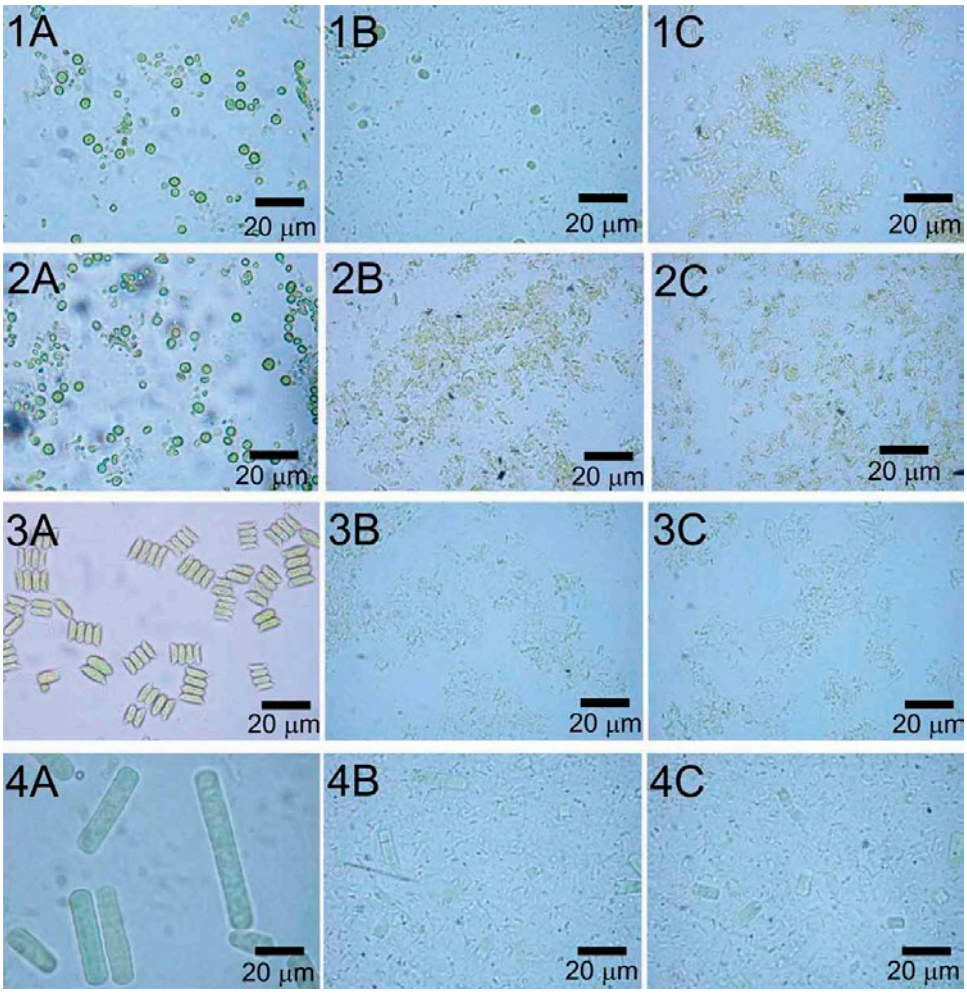

Figure 2 Whole-cell protein fingerprints of blue-green alga (Sp – Spirulina platensis) and green freshwater microalgae (ChK – Chlorella kessleri; Sc – Scenedesmus quadricauda; Ch – Chlorella sp.) after autotrophic cultivation in solar photobioreactor (AP), autotrophic cultivation in outdoor open circulating cascade-type cultivation apparatus in thin-layer (AO) and after treatment by oscillatory globe mill (M), Balotina B7 (B) in molecular weight (kDa). Standard is noted as S; molecular weight standards: 116; 97.2; 95.3; 66.4; 55.6; 27; 20 and 14.3 kDa.

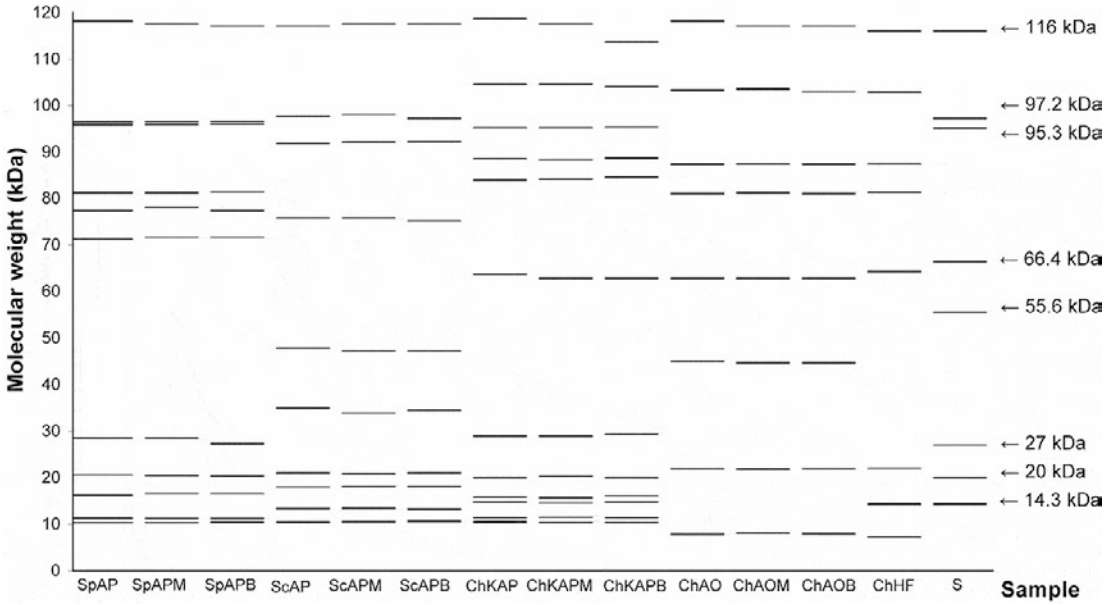

Figure 3 The dendrogram with homology of blue-green alga (Sp – Spirulina platensis) and green freshwater microalgae (ChK – Chlorella kessleri; Sc – Scenedesmus quadricauda; Ch – Chlorella sp.) after autotrophic cultivation in solar photobioreactor (AP), autotrophic cultivation in outdoor open circulating cascade-type cultivation apparatus in thin-layer (AO), heterotrophic cultivation in fermenter (HF) and after treatment by oscillatory globe mill (M), Balotina B7 (B). The dendrogram was calculated by the UPGMA clustering method using Dice coefficients.