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Improvement of Nutrition Production by Protoplast Fusion Techniques in *Chlorella vulgaris*

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Abstract

Recent decades showing remarkable development of the biotechnology of microalgae. Valuable product for food, nutrition and other applications will extend into broader area. Natural nutrition production from microalgae are not yet competitive with their synthetic levels. *Chlorella* is widely used as a health food and feed supplement, as well as in the pharmaceutical and cosmetics industries. Protoplast fusion was found to be an efficient method in improving its nutrition production and diversification in *Chlorella vulgaris*. The research was carried out by application of protoplast fusion on interspecific microalgae of *C. vulgaris*. The fusant was subjected for analysis of nutrition content by GCMS methods on *C. vulgaris* powder from 100 L liquid cultivation of fusant. The research result gained fusant in high mass production level. Nutrition analysis of fusants showed 17 amino acid with high concentration glutamic acid (14495.52 ppm) followed by leucine (10856.97 ppm) and Aspartic acid (10378 ppm). Palmitic acid (1.59%) was showed highest concentration in its lipid acid profile. Lipid analysis also showed polyunsaturated fatty acids (PUFA) with concentration 1.0987% and DHA 0.2%. Surprisingly, the fusant also revealed Omega 9 instead of Omega 3 and Omega 6. The research result showed potential acquisition of improvement nutrition by protoplast fusian application on microalgae.

Keywords: Microalgae; Nutrition; *Chlorella*; Protoplast fusion

Introduction

Microalgae represent the largest natural nutrition in the aquatic food chain of animal aquaculture. *C. vulgaris* was one of the source of aquatic nutrition [1]. This species have been exploited for aquaculture feeds as therapeutic or nutritional supplements [2]. Production of genetically improved strain by somatic fusion and hybridization on algae has been reported in some algae [3]. Protoplast fusion has been conducted inter and intraspecies on several genus of microalgae [4]. However, microalgae are still not a well-studied group from a biotechnological point of view. The genetic improvement of algal strains is also a present challenge to improve the potency of microalgae. Algal production systems need to be further improved to become more competitive and more economically feasible.

Protoplast fusion was *in vitro* genetic manipulation techniques which are more effective compared with conventional techniques used for strain improvement like mutation and selection [5-9]. Somatic hybridization allowed by this technique has been proven effective increasing nutrition and valuable metabolites production [10]. Combination of fully or partially nuclear and cytoplasmic genomes levels also enables to performed using protoplast fusion. Microalgae are very rich in nutrition. Indeed, some nutrition are of major importance for many marine animals for the growth and metamorphosis of many larvae [7-15]. Application of protoplast fusion process for microalgae *Chlorella* had conducted to improve their carotenoid for animal aquatic supplement [10]. The use of fusant as a natural feed supplement is potential to overcome synthetic feed [1,16-19]. Application of fusant as natural feed supplement on *Penaeus monodon* post larvae had raised the weight growth, and survival from microbial diseases and immune response in wide range of salinity [1,20].

Protoplast fusion on algae algae have been reported as a valuable process to improve their nutrition. However, there are inconclusive reports about their effect of nutrition value of microalgae algae and the bioavailability of the nutrition within. More research is needed to investigate the effect of protoplast fusion for the nutrititonal value in *C. vulgaris in vivo*. The purpose of this study is to improve the of nutrition production on *C. vulgaris* using protoplast fusion techniques.

Materials and Methods

Chlorella vulgaris cultivation

The *C. vulgaris* was the microalgae originally from Brackish Water Aquaculture Development Centre (BBPBAP) on Jepara, Indonesia. They were grown in seawater tanks with the room temperature, recirculated and aerated in salinity at 25% to 30%. The microalgae were cultivated using sea water enriched with Walne media.

Microalgae media

Walne media for microalgae growth and cultivation consists of $FeCl_3$ 0.15 gL^{-1}, $NaNO_3$ 10 gL^{-1}, Na_2EDTA 45 mgL^{-1}, NaH_2PO_4 20 gL^{-1}, H_3BO_3 3.36 gL^{-1}, $MnCl_2.4H_2O$ 0.36 gL^{-1}, trace metal solution 1 mLL^{-1}, and distilled water. Trace metal solution consists of $ZnSO_4.7H_2O$ 0.222 gL^{-1}; $NaMoO_4.5H_2O$ 0.39 gL^{-1}; $Co(NO_3)_2.6H_2O$ 0.0494 gL^{-1}; H_3BO_3 2.86 gL^{-1}; $CuSO_4.5H_2O$ 0.079 gL^{-1}; and $MnCl_2.4H_2O$ 1.81 gL^{1}; pH 6.8. The ingredients were dissolved in 200 mL of distilled water. The solution was adjusting the pH to 7.6 with HCl or NaOH while boiled for 10 min continued with filtering and brought to 1 L by distilled water. Sterilization was done using autoclave at 15 $lb.in^{-2}$ (103 kPa and 120°C). The medium was used by adding 10 mL solution to each 1 L of seawater [21,22].

Nutrition analysis

Nutrition analysis was conducted at Diponegoro University

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using GCMS method using. Gravimetry method was used to analyze moisture, ash, fiber crude and lipid. Macro-Kjeldhal method was used to determine protein. Analyzing of fatty acid and amino acid profile were conducted at PT. Saraswanti Indo Genetech, Bogor based on AOAC method [23]. Amino acids were determined by UPLC Method and fatty acids were determined by GC Method. Carbohydrates were analyzed utilizing a colorimetric technique.

GC-MS analysis

A gas chromatography-mass spectroscopy (GC-MS) technique was used to analyze the chemical composition of microalgae. The sample was mixed with 300 µl n-hexanePatchouli oil were separated on a 30 m × 0.25 mm (l × i.d.) capillary column coated with a 0.25 mm film of 5% phenyl methyl siloxane at a column temperature of 80°C for injection. Temperature programming started at 10°C min^{-1} to 150°C and then from 5°C min^{-1} to 250°C and finally from 10°C min^{-1} to 280°C and held for 5 min. Using splitless injection, (2 µL), helium was employed as the carrier gas with a flow rate of 1 mL/min. The spectrometer was operated in electron-impact (EI) mode, with electron energy of 70 eV and a scan range of 50- 550 amu. The inlet and ionization source temperatures were 240°C and 280°C, respectively.

Protoplast fusion

Fusion of protoplast was conducted using modified methods of Tjahyono et al. [24] and Uppalati and Fujita [25]. Cells of microalgae with a density of 10^7 was soaked in a solution of sodium succinate buffer (pH 4.5), consist of 0.7 M $(NH_4)_2SO_4$, 0.6 M KCl and 0.1 M 2-mercaptoethanol. The protoplast is obtained by suspending the microalgae cells into 2-3 mg mL^{-1} lysozyme for 2-3 hours. Early growth phase of protoplast (approx.10^7-10^8 cells mL^{-1}) were washed with osmotic solubilizing solution (potassium phosphate buffer) followed by its suspension in 3% sodium chloride buffer, 1 mM $CaCl_2$ and 0.1 M 2-mercaptoethanol. The protoplast was resuspended in 1% 10 mg mL^{-1} of lysozyme on 35°C for 20 minutes. The protoplast of *Chlorella* was kept in Walne medium for 45 min. Composition of Walne medium was sea water, 60 mM polyethylene glycol (Mr.6000; Sigma), 5 mM glycine and 10 mM $CaCl_2$. The process was followed by serial washing in suspension containing 5 mM glycine and 10 mM $CaCl_2$. Protoplast regeneration were made by growing the recombinant on Walne medium using sea water containing 5 mM glycine and 10 mM $CaCl_2$ and incubated for 5-7 days [26,27].

Results and Discussion

Protoplast fusion on *C. vulgaris*

Microalgae *C. vulgaris* has been already characerized using moleculer techniques before protoplast fusion process [28]. The protoplat fusion process had produced stable hybrids which in tend to combine more than one cell and create new cell wall as illustrated in Figure 1. We can also see that the combination of more than one cell will make the cell have a smaller size than its parent.

The *Chlorella* fusant are shown in Figure 1. The protoplast fusion result showed variation of cell combination. Each fusant consist of two or more cell that combine in one cell. These fusant were grown in salinity about 15%. Growth were analyzed for the fusant and were compared with the parental *C. vulgaris*. The growth rate analysis revealed significant differences between fusant and parental. The growth of *C. vulgaris* and fusant during cultivation share the same pattern for eight day. This pattern are in accordance with Chia et al. [26]. The observed growth phases consist of the lag phase, the exponential phase and the

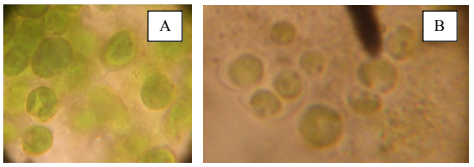

Figure 1: Protoplast fusion product (A: Parental *C. Vulgaris*; B: Fusant).

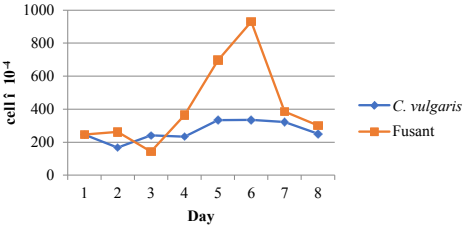

Figure 2: Growth of parental *C. vulgaris* and fusant.

No	Parameters	*C.vulgaris*	Fusant	Unit
1	Total energy	154.43	179.24	k-kal/100 g
2	Lipid energy	41.85	55..80	k-kal/100 g
3	Water	11.22	12.29	%
4	Ash	57.99	50.65	%
5	Total lipid	4.65	6.20	%
6	Protein	10.36	13.82	%
7	Total carbohydrate	15.78	17.04	%

Table 1: Proximate analysis of *Chlorella vulgaris* and fusant.

stationary phase. Both parental and fusant need adaptation on their growth which is marked with the reduction of cell count (Figure 2). The fusant showed faster growth comparing with the parental especially in the day sixth. The expermented microalage alo showed shorter growth comparing with study by Costard et al. [27] which is needed 12 days for *Chlorella* sp. growth.

Proximate analysis

Microalgae can be considered into organisms that producing a distinct range of chemical and biological compounds, principally vitamins, pigments, proteins, minerals, lipids and polysaccharides. The high nutrition content of microalgal species is one of the main reasons to consider them as an important source of nutrition for multipurpose target. This proximate composition of *C. vulgaris* and fusants are summarized in Table 1. Almost all of the proximate content of the fusant microalgae species tend bigger that their parent ranging from 15% to 25%. This result was in agreement with Gupta et al. [29] for the hybrid fom protoplast fusion between macroalgae *Monostroma oxyspermum* (Kutz.) Doty and *Ulva reticulata* Forsskål.

In this research, microalga cell harvesting was conducted in stationary phase to complete the process of adaptation and regeneration. The reduced growth seems affected the nutrition on microalgal cell exhibited by a low protein and higher carbohydrate content. These factor were determined in the study of Sharma et al. [30] which showed that lower degree of illumination will favors higher protein in *Chlorella*. *C. vulgaris* and fusant exhibited 5% to 6% total lipid content. Bi and He [31] reported that *C. vulgaris* has total lipid content varied from 30% to 60% depend on nutritional and growth conditions. However, the lipid content in this research was higher comparing with the study on *C. vulgaris* by Yusof et al. [32], and Torres et al. [33].

Protein content of *C. vulgaris* and fusant

Information on the protein value microalgae will provide their essential roles and development by application of protein supplement because protein content is a major factor determining the nutritional value of microalgae. The cells of *C. vulgaris* and fusant are capable of synthesizing almost all amino acids. Typical amino acid analysis of experimented microalgae is shown in Table 2. The presence of essential amino acids (ESA) such as arginine, phenylalanine, histidine, isoleucine, leucine, lysine, methionine, threonine, and valine was found in the profile of *C. vulgaris* and fusant, except. This result is almost the same with study by Samek et al. [34] which is found seven essential amino acid on *Chlorella*, while Safafar et al. [35] found all the amino acid. This research was not found tryptophan in the amino acid profile on both experimented microalgae. This result was confirmed by Volkman et al. [36], Bleakley and Hayes [15] which states that tryptophan and lysine are often limiting amino acids in most algae species.

Although Aspartic acid and Glutamic acid were non-essential amino acids (NESA) but they constitute the highest proportion of of the total amino acids among parental microalgae and fusant. This result was supported by Samek et al. [34], Bleakley and Hayes [15]. The result also showed some differences in ESA and NESA concentration with other microalgae like Samek et al. [34]. Cysteine have the lowest level of NESA in the investigated microalgae and fusant, as also typically occurs in many seaweed species [15]. However, experiment on *Spirulina platensis, Chlorella* sp, *C. kessleri,* and *S. quadricauda* showed that histidin having low level NESA [34]. However, total protein contents were directly proportionate to the growth and chlorophyll contents. The highest amount of protein was found in natural day light at 25° to 30° as also performed in this research.

The fatty acid profile of *Chlorella vulgaris* and fusant

Algal lipids are composed of glycerol, sugars or bases esterified to saturated or unsaturated fatty acids (12 to 22 carbon atoms). The results presented in Table 3 showed that *C. vulgaris* and fusant contain the Polyunsaturated Fatty Acids (PUFAs) include docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA), γ-linolenic acid (GLA), alpha linolenic acid (ALA) which have been widely recognized as beneficial towards human health. These results are similar to those observed by Yusof et al. [32]. This result was in contrast with Brown et al. [37], also Guedes and Malcata [19] which is stated that *Chlorella* spp. as a Chlorophytes are deficient in both C20 and C22 PUFAs. Moreover, *C. vulgaris* and fusant demonstrated high concentrations of Omega 3, as well as a a low concentration of Omega 6. Surprisingly, we also found moderate concentration of Omega 9. This result was different with Sayeda et al. [13] which was not detected omega 9 in *C. vulgaris*. Among all the fatty acids in microalgae, Omega 3 and Omega 6 families are of particular interest. In addition, highly unsaturated fatty acid (e.g., eicosapentanoic acid (EPA), arachidonic

S. no	Essential amino acids	*C. vulgaris*	Fusant	ppm
1	Leucine	8142.73	10856.97	ppm
2	Threonine	6113.47	8151.29	ppm
3	Lysine	5481.07	7308.09	ppm
4	Valine	5052.74	6736.99	ppm
5	Phenylalanin	4701.26	6268.34	ppm
6	Isoleucine	4049.33	5399.10	ppm
7	Methionine	1647.96	2197.28	ppm
8	Arginine	5071.65	6762.20	ppm
9	Histidine	1386.05	1848.07	ppm
S. no	**Non-essential amino acids**	***C. vulgaris***	**Fusant**	**ppm**
1	Glutamic acid	10871.64	14495.52	ppm
2	Aspartic acid	7783.52	10378.02	ppm
3	Alanine	6121.63	8162.17	ppm
4	Glycine	5327.34	7103.12	ppm
5	Serine	4724.26	6299.01	ppm
6	Proline	3569.25	4759.00	ppm
7	Tyrosine	2720.49	3627.32	ppm
8	Cysteine	127.59	170.12	ppm

Table 2: Content of amino acids of *Chlorella vulgaris* and fusant.

S. no	Lipid acid	*C. vulgaris* (%)	Fusant (%)
1.	C 4:0 (Butyric acid)	0.0120	0.0147
2.	C 6:0 (Caproic acid)	0.0020	<0.0013
3.	C 8:0 (Caprylic acid)	0.0200	0.0267
4.	C 10:0 (Capric acid)	0.0264	0.0339
5.	C 11:0 (Undecanoic acid)	0.0014	<0.0016
6.	C 12:0 (Lauric acid)	0.1496	0.1988
7.	C 13:0 (Tridecanoic acid)	0.0657	0.0862
8.	C 14:0 (Myristic acid) SFA	0.3981	0.5295
9.	C 14:1 (Myristoleic acid)	<0.0014	<0.0017
10.	C 15:0 (Pentadecanoic acid) SFA	0.0258	0.033
11.	C 15:1 (Pentadecenoic acid)	<0.0013	<0.0016
12.	C 16:0 (Palmitic acid) SFA	1.19730	1.5964
13.	C 16:1 (Palmitoleic acid) MUFA	1.05985	1.4131
14.	C 17:0 (Heptadecanoic acid)	0.0239	0.0317
15.	C 17:1 (Heptadecenoic acid)	<0.0015	<0.0016
16.	C 18:0 (Stearic acid) SFA	0.1298	0.1729
17.	C 18:1 (Oleic acid) MUFA	0.41239	0.5505
18.	C 18:2 (Linoleic acid)PUFA	0.1040	0.1387
19.	C 18:3 (Linolenic acid)PUFA	<0.0012	<0.0015
20.	C 18:3 (Linolenic acid/ALA)PUFA	0.0246	0.0315
21.	C 20:0 (Arachidic acid) SFA	0.0186	0.0234
22.	C 20:1 (Eicosenoic acid)	<0.0012	<0.0015
23.	C 20:2 (Eicosedienoic acid)	<0.0013	<0.0015
24.	C 20:3 W3(Eicosatrienoic acid)	<0.0012	<0.0016
25.	C 20:3 W6(Eicosatrienoic acid)	<0.0015	<0.0016
26.	C 20:4 W6 (Arachidonic acid/AA)	0.1508	0.2010
27.	C 20:5 W3(Eicosapentaenoic acid/EPA)Omega 3	0.8250	1.0987
28.	C 21:0 (Heneicosanoic acid)	0.0179	0.0236
29	C 22:0 (Behenic acid)	<0.0014	<0.0014
30.	C 22:1 (Erucic acid)	<0.0011	<0.0015
31.	C 22:2 (Dokosadienoic acid)	<0.0012	<0.0016
32.	C 22:6 (Docosahexaenoic acid/DHA)	<0.0019	<0.0023
33.	C 23:0 (Tricosanoic acid)	<0.0012	<0.0014
34.	C 24:0 (Lignokeric acid)	<0.0012	<0.0016
35	Omega 3	<0.9475	<1.25
36	Omega 3 total	0.8240	1.0987
37	Omega 6	0.1050	0.1387
38	Omega 6 total	0.2785	0.3713
39	Omega 9	0.4139	0.5505
40	Omega 9 total	0.4129	0.5505
41	Unsaturated fatty acid	2.59520	3.4336
42	Saturated fatty acid	2.07883	2.7715
43	Monounsaturated fatty acid (MUFA)	1.48270	1.9636
44	Total Polyunsaturated fatty acid (PUFA)	1.10443	1.4699
45	AA	0.1508	0.2010
46	DHA	<0.0009	<0.0012
47	PUFA	0.8240	1.0987

AA: Arachidonic Acid; DHA: Docosahexaenoic Acid; SFA: Saturated Fatty Acid; MUFA: Monounsaturated Fatty Acid; PUFA: Polyunsaturated Fatty Acid

Table 3: Fatty acid acid profile of *Chlorella vulgaris* and fusant.

Variables	Compound name	*C.vulgaris* (% area)	RT	Fusant (% area)	RT
Omega 6	Hexadecenoic acid, 2-hydroxy-1,3-propanediyl ester (CAS)	8.60	48.021	13.61; 4.41	48.010 52.115
	Hexadecenoic acid, methyl ester (CAS)	1.97	39.982		
	9,12-Octadecadienoic acid (Z, Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (CAS)	30.62	51.384	26.47	51.381
Omega 9	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester (CAS)	--	--	55.43	51.576
	9-Octadecenoic acid (Z)-, 2-(acetyloxy)-1- [(acetyloxy)methyl] ethyl ester (CAS)	46.62	51.562	--	--
	9-Octadecenoic acid (Z)-, methyl ester (CAS)	2.39	43.989	--	--
	14-BETA-H-PREGNA	3.35	51.680	--	--

Table 4: GC/MS chemical profile of *C. vulgaris* and fusant omega fatty acids ester.

acid (AA) and docosahexaenoic acid (DHA) also found in *C. vulgaris* and fusant. Because of its role as a producer in the food chain at sea, microalgae also potentially being a source of polyunsaturated fatty acids (PUFA), δ-linoleic acid (GLA). The total amount and relative proportion of fatty acids can be affected by nutritional and environmental factors, nitrogen limitation. We also found C16:0, C16:1, C18:1 C18:2 and C18:3 which have not been reported before for *C. vulgaris* (Table 3). The existence of the erucic acid methyl ester (C22:0), arachidic acid methyl ester (C20:0), palmitic acid methyl ester (C16:0), cis-11-eicosenoic methyl ester (C20:1), cis-11, 14-eicosadienoic acid methyl ester (C20:2) and linolenic acid methyl ester (C18:3) might be potencial as antimicrobial agent based on the experiment conducted by Suresh et al. [11]. The fatty acid composition of green alga *Chlorella* of 14:0, 16:0, 16:1, 16:2,16:3, 18:0, 18:1, 18:2, 18:3 is confirmed under all kinds of cultivation conditions as those mentioned by Wang et al. [7].

Our research as exhibited in Table 4. also showed the natural occurrence of Fatty acid ester in *C. vulgaris* and fusant. Major fatty acid esters were C16:0 and C18:1 (Table 3), in agreement with a previous work on *C. reinhardtii*. The hexadecanoic acid also dominant for macroalgae, *Himanthalia elongata* [9]. Octadecadienoic acid was the most abundant fatty acid under laboratory conditions for both *C. vulgaris* and fusant. Hexadecanoic acid was the second abundant fatty acid. Both acids increased in their contents in the fusant. It is also found that the fusant also tend to have the same retention time with their parental for similar compound of fatty acid. Surprisingly, fusant and their parental *C. vulgaris* producing different type of Omega 9 compound in almost the same time retention.

Based on the research, it is showed that protoplast fusion process could be enhanced all of the nutritional value af *C. vulgaris*. This process also could create a new compound that can be found in their parental like 14-BETA-H-PREGNA fatty acid. The nutritional value of fusant from *C. vulgaris* not only exhibited a similar trend with their parental but also tend to increase and vary more nutritional compound.

Conclusion

Protoplast fusion are a method for improving nutrition which show great promise, but have yet to be investigated sufficiently in microalgae. In this work, application of protoplast fusion process and their effect on the nutritional value in *C. vulgaris* is presented. This tehcniques not only allows to increased the original compounds found and also vary the nutritional compound. It can be considered as a powerful tool in microalgae. The possibility to find functional interesting compounds from microalgae using this techniques looks promising.

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