Enzyme-assisted extraction of fatty acid from *Caulerpa lentilifera*: a preliminary study

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Abstract. Polyunsaturated fatty acids (PUFA) are essential nutrients that play an essential role in the functioning of the brain and cell growth. Mammals, including human, cannot synthesize PUFA. Therefore, the intake of PUFA must be obtained from dietary sources. Marine macroalgae, such as *Caulerpa lentilifera*, are excellent sources for high PUFA. The extraction of PUFA in the pure form is usually carried out by conventional extraction whereas not environmentally friendly. Therefore, using enzymatic-assisted extraction is a great alternative to obtain PUFA from *Caulerpa* sp. with lower cost and fewer usage of solvents. In this research, we examined the effectivity of enzyme-assisted extraction using papain enzyme because its cheap and easy to find. The optimum extraction method to obtained bioactivity from lipid extract of *C. lentilifera* by enzyme assisted extraction using papain enzyme was 0.5% concentration of papain, temperature 60˚C for 16 hours incubation.

Keywords: *Caulerpa lentilifera*, enzyme-assisted extraction, papain, PUFA

1. Introduction

Algae are one of the essential bioresources from coastal that have been used as food resources and the extract used as many industrial chemicals for daily human needs (Kumari *et al* 2013). In recent years, the trend of nutrition supplement to fulfill the quality of human intake was increasing. Notably, interest in food supplement from natural resources is rise because synthetic agents have been suspected induce damaged for human health (Farag *et al* 2013). Thus far, algae with many utilizations are mainly red and brown algae. On the other hand, green algae are used as raw food for salad or coated by sugar, especially in east Indonesia. The culture of green algae has been done by Brackish Aquaculture Fisheries Center, Takalar, South Sulawesi, Indonesia. The resulted algae is used for community consumption, also for export to Japan.

One of the green algae that have been cultured is *C. lentilifera*, which is a green alga that has bioactive compound such as polyunsaturated fatty acid, antidiabetic agent, anti-cardiovascular agent, and many others. *C. lentilifera* are widely consumed by people in Japan and southeast Asian countries. *C. lentilifera* generally grows on sandy rock bottoms in the upper sublittoral coral reefs tropical zone (Mao
et al 2011). The culture of Caulerpa gives many benefits because of its low investment, high profitability, restore the usage of a pond, and give a better water quality that is good for the environment. C. lentillifera contain high amount of mineral such as phosphorus, magnesium, manganese, potassium and calcium. It is also rich in vitamin E, vitamin A and polyunsaturated fatty acids (PUFA) such as linoleic, linolenic, palmitoleic, and eicosanoic acids. Moreover, the methanolic extract of green algae C. lentillifera resulted better reducing power for 362.11±15.65 µM/mg dry extract, and radical-scavenging activity for 2.16±0.04 mM Trolox equivalents/mg dry extract, also higher phenolic content for 42.85±1.22 mg Phloroglucinol equivalent/g dry extract, than the other algae (Matanjun et al 2008). Matanjun et al (2009) reported the crude protein and the PUFA contents of C. lentillifera was 10.41% DW and 16.76%, respectively. Furthermore, mainly PUFA cannot be synthesized by the human body, so human need to consume food as the source of PUFA. Accordingly, PUFA in C. lentillifera potential as a diversification product and very interesting to be explored.

Extraction technology of lipid, especially PUFA, need several organic solvents, which not good for our environment. So that we need another extraction method that can extract lipid and also suitable for the environment or “green technology”. One of the green technology that we can use is enzyme assisted extraction. Enzyme-assisted extraction is an eco-friendly, solvent-free, and cost-effective extraction method. An enzyme is an ideal catalyst to improve extraction yield, synthesis and modification of bioactive compound from natural origin. The useful application of enzymes could increase the solvent pre-treatment effects, and either could reduce the amount of solvent needed for extraction, also could increase the yield extract of active compounds (Puri et al 2012).

Moreover, enzymes could disrupt or degraded cell walls and membranes, that can make better release and increasing the efficiency of bioactive extraction Pinelo et al (2006). However, the purified enzyme is costly, so we need an alternative for an enzyme with an affordable price. One of an affordable enzyme is papain enzyme, its proteolytic enzyme that found naturally in papaya (Carica papaya L.) and produced from the latex of raw papaya (Amri and Mamboya 2012). Considering the papaya grows in an almost whole year and a wide range of climate, make papain easy to find and cheap. Furthermore, the use of papain enzyme is effortless, very stable even at elevated temperatures and have an optimum pH in the range of 3.0-9.0, which varies with a different substrate (Cohen et al 1986, Ghosh 2005). Therefore, this research has purposes of doing the optimization of PUFA extraction method using enzyme assisted extraction by papain enzyme on C. lentillifera. Also, we analyzed the bioactivities of the resulted lipid extract by determining antioxidant activities using ABTS assay. Last, the profiling of PUFA in resulted lipid extracts has been done by using GCMS.

2. Materials and methods

2.1. Sampling
C. lentillifera was taken from Takalar, South Sulawesi, Indonesia, which that area is specialist on Caulerpa culture. The sample preparation after the shipment was rinsed with tap water then dried by aerated. After that, the sample was blended slowly to make them into a slurry then weighed for the extraction and next treatments.

2.2. Lipid extraction
The slurry of all the samples was weighed for each 100 mg added into 250 mL Erlenmeyer. Then the samples were sonicated for 15 minutes on cold condition. Determination of pH was done before the addition of enzyme. To reach the optimum of the papain enzyme, added 1 mol/L NaOH until pH 7. Then the addition of enzyme for the concentration of 0.5% (wet basis). For activation of the enzyme, all the samples were incubated in a water bath with treatments of temperature 40°C and 60°C for 30 minutes. After that, the samples were fermented by shaking in room temperature for 1.5 hours and 16 hours. Then after the fermentation treatments finished, inactivated the enzyme by soaking in a water
bath using temperature 85°C for 10 minutes. The samples were cooled down and prepared for lipid extraction using dichloromethane.

Lipid extraction was using Bligh and Dyer method (1959) by chloroform, but for proposed of the making of food products and to make the products safer consequently the extractions were done using dichloromethane. The solvent was used 1:1 (v/v) with the samples. The filtrate of the sample was separated using filter paper. Dichloromethane was added to the filtrates and put in the separating funnel to the separated aqueous and organic phase. The extraction using dichloromethane were done three times to make sure all lipid content was extracted. After that, the lipid extracts in the organic phase were concentrated using rotary evaporator until we can obtain dried extract for the calculation of extract yield.

2.3. Bioactivity analysis using ABTS

Bioactivity analysis was done by determination of antioxidant activities using the ABTS method (Re et al 1999). Radical ABTS solution was prepared the day before, by mixed 5 mL of 19.2 mg ABTS (2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) with 88 µL 140 mM potassium persulfate in distilled water and kept for 12-16 h. In the next day, mixed solvents of ABTS+ were diluted using 99% of EtOH until 200 mL that we can use for maximum next three days. Control solvent was prepared using 99% EtOH. Determination of antioxidants was begun by incubated 1 mL of ABTS+ and 1 mL extracts or standard for 6 minutes at 37°C in a water bath. The antioxidant activity was measured using spectrophotometer in absorbance 734 nm. Inhibition of ABTS+ calculated based on IC50 value using the equation as below:

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\text{Percentage of ABTS radical scavenger activities} = \left(\frac{A_{con} - A_{test}}{A_{con}}\right) \times 100\%
\]

*Where A_{con} is controlling absorbance and A_{test} is extracting absorbance

2.4. Profiling of fatty acid using GCMS

Fatty Acid Methyl Ester (FAMEs) was done by using KOH (Gao et al 2013). The convert of fatty acid in the lipid extracts become FAMEs was done by added 2 mL of KOH-CH3OH (0.4 mol/L) and heated for 40°C for 10 minutes in a water bath. Afterward, FAME’s were extracted using 2 mL of n-hexane. The mixture was added with 5 mL water, then shaken vigorously for 15 s and left to stand. The upper layer containing FAMEs was formed then transferred into a 2 mL autosampler vial for determination into GCMS.

The GCMS condition for this research was:

| Column Oven Temperature: 60°C | Oven Temp. Program: |
|-----------------------------|-------------------|
| Injection Temperature: 300°C | - Temp 115°C for 2 min |
| Pressure: 14.6 psi | - 10°C/min increased to 200°C |
| Total Flow: 45 mL/min | - Temp 200°C for 18.5 min |
| Column Flow: 1 mL/min | - 60°C/min increased to 245°C |
| Linear Velocity: 29 cm/sec | - Temp 245°C for 4 min |

3. Results and discussion

The highest lipid extract yield, 73.9 mg/100 g wet basis, was obtained by incubation of papain enzyme 0.5% at 60°C for 30 minutes and fermentation in 1.5 hours (figure 1). In opposite the lowest lipid extract yield, 6.1 mg/100 g wet basis, was obtained by the same concentration and same temperature but the time is very long that was 16 h. Those condition suspected due to papain enzyme has optimized on degrading the cell wall in *C. lentilifera* on 60°C for 30 minutes and fermentation in 1.5 hours. However, if the fermentation time stays longer, the lipid extract decreased.
Extraction is the crucial step in isolating various types of bioactive compounds from bioresources, including algae. Ideally, the extraction methods expected to be quantitative and time-saving. Enzymes have been used mainly for the treatment of plant material, particularly prior to conventional methods of extraction such as maceration and distillation. The enzyme used to disrupt the structure of the plant cell wall, that could enhance the extraction of various bioactive compounds inside the plant. The enzymes such as cellulose, hemicellulose and many others, used for hydrolyzing cell wall components so it can increase cell wall permeability, consequently in higher extraction yields of bioactive compounds (Puri et al 2012).

**Figure 1.** Lipid extract yield of *C. lentilifera* using enzyme assisted extraction by papain enzyme. C4h15: incubation in 40°C fermentation for 1.5 hours; C4h16: incubation in 40°C fermentation for 16 hours; C6h15: incubation in 60°C fermentation for 1.5 hours; C6h16: incubation in 60°C fermentation for 16 hours. Bars with different letters are significantly different (p<0.05).

Particularly in oil or lipid extraction, it is known that the used of enzyme produces a higher content of antioxidant compounds in oil extract from olive oil (Garcia et al 2001). Moreover, Soto et al (2008) reported that a defatted meal of evening primrose and borage oil also give better result by adding an enzymatic treatment. The quality of oils extracts obtained by enzyme treatment resulted a better extract compared with oil extraction using hexane. Thus, an ideal alternative of green technology for seed oil extraction by using enzyme-assisted cold pressing (EACP) because of its non-flammable and non-toxic properties.

The highest antioxidant activities were obtained by papain enzyme 0.5% at 60°C for 16 hours (table 1). Even though the yield of that treatment was the lowest among all the treatments, but it obtained the highest activities as an antioxidant, so we decided to analyzed lipid extract profiling using GCMS. Free radicals are involved in lipid peroxidation, which has a vital role in the development of many diseases such as cancer, heart disease, and Alzheimer’s (Halliwell and Gutteridge 1989). The ability to scavenge free radicals is a principal antioxidant property (Mohadjerani 2014). However, further analysis of the antioxidative system is needed to identify the role individual compounds as antioxidant.

**Table 1.** Antioxidant activities by ABTS Method.

| Enzyme conc. (%) | Temp. (°C) | Time (h) | IC50 (ppm) |
|------------------|------------|----------|------------|
| 0.5              | 40         | 1.5      | 474<sup>a</sup> |
| 40               | 16         |          | 1445<sup>c</sup> |
| 60               | 1.5        |          | 4640<sup>d</sup> |
| 60               | 16         |          | 78<sup>a</sup> |
| Vit. C (as standard) |          |          | 2.8        |

Number with different letters are significantly different (p<0.05).

GCMS analysis showed that the main active compound in lipid extract was from a derivative of hexadecenoic acid and octadecadienoic acid or known as omega 9 (table 2). However, we could not
detect omega 3 and 6 in the extract. For further investigation, enzyme assisted extraction for PUFA in *C. lentilifera* still need to explore such as for the uses of enzymes and how to extract lipid particularly PUFA.

| Bioactive compound                        | Peak area (%) | Activity                          |
|-------------------------------------------|---------------|-----------------------------------|
| Hexadecanoic acid, methyl ester           | 5.24          | Anti-inflammatory, antioxidant, decreased blood cholesterol |
| 9,12-Octadecadienoic acid (Z,Z)-, methyl ester | 7.24          | Anticancer                        |
| 9-Octadecenoic acid, methyl ester         | 3.87          | Antioxidant, anticancer           |

4. Conclusion

The optimum extraction method to obtain bioactivity from lipid extract of *C. lentilifera* by enzyme assisted extraction using papain enzyme was 0.5% concentration of papain enzyme, temperature 60°C for 16 hours incubation. In the next future pathway of the enzyme to the degraded cell wall of *C. lentilifera* need to be explored, so the optimum condition of enzyme assisted extraction could be reached.

References

Amri E and Mamboya F 2012 Papain, a Plant Enzyme of Biological Importance: A Review *American Journal of Biochemistry and Biotechnology* 8 99-104

Bligh E G and Dyer W J 1959 A rapid method of total lipid extraction and purification *Can. J. Biochem.Physiol.* 37 911-917

Cohen L W, Coghlan V M and Dihel L C 1986 Cloning and sequencing of papain-encoding cDNA *Gene* 48 219-227

Farag RS, El-Baroty and Basuny M 2003 Safety evaluation of olive phenolic compounds as natural antioxidant *Int J Food Sci Nutr* 54 321-326

Gao Y, Yang M and Wang C 2013 Nutrient deprivation enhances lipid content in marine microalgae *Bioresour. Technol.* 147 484-491

Garcia A, Brenes M, Moyano M J, Alba J, Garcia P and Fernandez AG 2001 Improvement of phenolic compounds content in virgin olive oils by using enzymes during malaxation *J. Food Eng.* 48 189-194

Ghosh S 2005 Physicochemical and conformational studies of papain/sodium dodecyl sulfate system in aqueous medium *J. Colloid Surf. A: Phys. Eng. Aspects* 264 6-16

Halliwell B, Gutteridge J M C 1989 *Oxidative stress: adaptation damage repair and death, Free radical in biology and medicine* Halliwell B, Gutteridge J M C Ed (Oxford: Clarendon Press) p 285-295

Kumari P, Bijo A J, Mantri V A, Reddy C R K and Jha B 2013 Fatty acid profiling of tropical marine macroalgae: an analysis from chemotaxonomic and nutritional perspectives *Phytochemistry* 86 44-56

Mao S C, Liu D Q, Yu X Q and Lai X P 2011 A new polycetylene fatty acid and other secondary metabolites from the Chinese green alga *Caulerpa racemosa* (Caulerpaceae) and their chemotaxonomic significance *Biochem Syst. Ecol.* 39 253-257

Matanjun P, Mohamed S, Mustapha N M, Muhammad K and Ming C H 2008 Antioxidant activities and phenolics content of eight species of seaweeds from north Borneo *J. Appl. Phycol* 20 367-373

Matanjun P, Mohamed S, Mustapha N M and Muhammad K 2009 Nutrient content of tropical edible seaweeds, *Eucheuma cottonii, Caulerpa lentillifera* and *Sargassum polycystum* *J. Appl. Phycol.* 21 75-80
Mohadjerani M, Tavakoli R and Hosseinzadeh R 2014 Fatty acid composition, antioxidant and antibacterial activities of Adonis wolgensis L. extract Avicenna J Phytomed. 4 24-30
Pinelo M, Arnous A and Meyer A S 2006 Upgrading of grape skins: the significance of plant cell-wall structural components and extraction techniques for phenol release Trends Food Sci. Technol. 17 579-590
Puri M, Sharma D and Barrow C J 2012 Enzyme-assisted extraction of bioactive from plants Trends in Biotechnology 30 37-44
Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C 1999 Antioxidant activity applying an improved ABTS radical cation decolorization assay Free Radical Biology And Medicine 26 1231-1237
Soto C, Concha J and Zuniga M E 2008 Antioxidant content of oil and defatted meal obtained from borage seeds by an enzymatic-aided cold pressing process Proc. Biochem. 43 696-699