Iranian Journal of Fisheries Sciences DOI: 10.22092/ijfs.2021.124430

Research Article

Optimization of production of enzymatic protein hydrolysatebased flavor from sea grape (*Caulerpa racemosa*)

Amin M.N.G. ^{1,2*}; Rizky Distiawan M.²; Herlina M.²; Cholifatun R.²; Rohim F.N.²; Anggraeni C.D.³; Alamsjah M.A.¹; Wahju T.¹; Sri S.¹

Received: February 2021 Accepted: March 2021

Abstract

Caulerpa racemosa (sea grape) is an abundantly growing algae in Indonesia which contains high amount of protein and has a seafood-like flavor. This species is not widely used by people due to the lack of information on its functionalities. Therefore, this study was objected to optimize the production of protein hydrolysate based flavoring agent by using a response surface methodology. The ratio of bromelain enzyme to substrate of 21.72, 30, 50, 70, 78.28 % w/w and hydrolysis time of 0.17, 1, 3, 5, 5.83 h was designed using a central composite design by developing a quadratic model to yield the highest protein yield. The air-dried sea grape was preincubated and poured with bromelain enzyme, incubated at 50°C, followed by heating at 95°C to terminate the enzymatic reaction. The hydrolysate was collected by centrifugation, and then filtered. This study revealed that the valid optimal solution of protein hydrolysis was obtained as the ratio of bromelain enzyme to substrate of 78.28% and hydrolysis time of 3.49 h with 0.3215% (w/w) yield. The present study showed that hydrolysis could release the volatile compounds originated from air-dried seaweed and even formed new products, such as hydrocarbon, aldehyde, alcohols, ketone, and fatty acid, which were resulted by thermal degradation of fatty acid during hydrolysis and Maillard reaction. By inferring with prior studies of sea food flavor, this study suggested that sea grape protein hydrolysate enzymatically produced by bromelain was highly potential as sea food flavoring agent.

Keywords: Caulerpa racemosa, Hydrolysis, Bromelain, Central composite design

¹⁻Department of Marine, Faculty of Fisheries and Marine, Universitas Airlangga, Mulyorejo, Surabaya, East Java 60115 Indonesia

²⁻Undergraduate Program of Fisheries Product Technology, Faculty of Fisheries and Marine, Universitas Airlangga, Mulyorejo, Surabaya, East Java 60115 Indonesia

³⁻Undergraduate Program of Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Mulyorejo, Surabaya, East Java 60115 Indonesia

^{*}Correspondence author's Email: m-nurghoyatulamin@fpk.unair.ac.id

Introduction

(colloquially Caulerpa racemosa known as "sea grape") is one of the abundantly growing green algae species in the upper sublittoral zone of tropical coral reefs such as Philippines, Vietnam. Singapore, Malaysia, Thailand, Taiwan, China, Indonesia, and West Pacific Coast (Horstmann, 1983). It is green in color, and smells like seafood; it has a relatively high protein content (17.80-21.73%) with a dominant residue of L-threonin, L glysine, L-glutamic acid and L-lysine amino acid (Pereira, 2011; Ma'ruf et Interestingly, al., 2013). characteristics are quite similar to other edible seaweeds such as Undaria pinnatifida, Porphyra tenera and Ulva lactuca which are highly commercial in Japanese food market due to their sea food flavor and umami taste (Kajiwara et al.. 1993; Fleurence, 2016). Traditionally, wild sea grape consumed fresh by coastal people in Indonesia and Malaysia. Due to the potential benefits of this species as food stock, the culture of this species has begun in the 1950s. In Indonesia, floating culture is the most appropriate method of culturing sea grape (Nuraini, 2006); Indonesian Seaweed Farmer Association and the government have developed a guideline for sea grape culture to ensure sustainable supply.

Study of Laohakunjit *et al.* (2014) and Qi *et al.* (2017) demonstrate that protein hydrolysis of byproducts of *Gracilaria verucosa* agar and *Undaria pinnatifida* sporophyll release flavoring

compounds. Also a study of Dang et al. (2015)stated that the protein hydrolysate contains low weight molecular peptides and free amino acids which could contribute to the umami taste. However, the use of byproducts could be inefficient, due to the lack of consistency in the remaining protein level from different production batch. Therefore, another source of algal protein and sea food flavor must be explored.

Protein hydrolysate could be obtained either chemical or enzymatic hydrolysis, although hydrolysis relatively enzymatic is easily preferable because it is controlled, efficient, reproducible, ecofriendly, and nontoxic (Chalamaiah et al., 2012; Arshad *et al.*, 2014). Bromelain derived from pineapple stems and fruit body of pineapple (Ananas comosus) is one of the foodgrade proteases. The enzyme generated by different parts of pineapple has different characteristics. The optimum pH for stem bromelain is alkaline while for fruit bromelain is acidic (de Lencastre et al., 2016). Stem bromelain has a wide specificity; and is active in alkaline condition at the optimum temperature of 50°C (Sarkar et al., 2017) and has been applied in the hydrolysis of algal biomass proteins (Laohakunjit et al., 2014).

A prior study by Nasri (2017) demonstrates that to optimize the process, hydrolysis conditions must be appropriately set up in terms of time, temperature, pH and enzyme to

substrate ratio (E/S). Response surface methodology (RSM) has been applied in various experimental designs on bioactive compound extraction (Huang et al., 2008) and for protein hydrolysis of Gracillaria sp. (Laohakunjit et al., 2014). The main advantage of using RSM is reduction number of trials needed to improve parameters (independent variables) and mutual interactions in the response process (dependent variable) (Rój et al., 2015).

In our preliminary work we have demonstrated that reaction time and E/S ratio were crucial factors in maximizing the water-soluble protein yield in the hydrolysis of *Caulerpa racemosa*. Therefore, the objective of the present study was to optimize the bromelain enzyme to substrate ratio and reaction time to maximize the yield of water-soluble proteins.

Materials and methods

Materials

Caulerpa racemose (sea grape) was harvested from the Brackish Water Aquaculture Center Jepara, Central Java (6°35'11.9"S 110°38'39.8"E) in October 2018. The bromelain enzyme with activity of 1382 GDU/g was supplied by PT. Bromelain Enzyme BR1200-I14611, Lampung. Lowry reagents were prepared by mixing 50:1 of 2% Na₂CO₃ in 0.1 N NaOH and 0.5% CuSO₄ in 1% Na Tartrate. Folin-Ciocalteu reagent was purchased from Merck, Germany. Cellulose filter paper No.1 was supplied by Whatman UK.

Preparation of air-dried sea grapes

Sea grapes were cleaned shortly after collection using freshwater to remove sand, debris, epiphytes, and other external matters, then placed in a box lined with banana leaf inside and transferred to the laboratory. Samples were fully air-dried at room temperature and stored in plastic bags with silica gel to control the storage environment. The protein content of sea grape in the present study was 17.85%.

Preparation of sea grape protein hydrolysate

Sea grape protein hydrolysate was produced as described by Laohakunjit et al. (2014) with slight modifications. Algae was mixed with distilled water at a ratio of 1:50 (2 g of algae in 100 mL of distilled water) followed by adjusting pH to 6 by dropping citric acid. The dispersion was then pre-incubated at 50°C for 10 min in the Memmert Universal Oven UF30. Germany. Enzymes were poured, the mixture stirred, and incubated. The reactions were terminated by heating the mixture at 95°C for 15 min and let cool to room temperature. **Hydrolysates** were centrifuged at 867 g for 15 min using a centrifuge (Hettich EBA 20, Germany). The supernatants were decanted and filtered through cellulose filter paper Whatman 01. The filtrates were collected in glass bottles, stored at -4°C for further analysis. All experiments were conducted in triplicate. The yields water-soluble of proteins were

determined according to the following

equation:

Chemical analysis

Protein content of algal sample was determined according to reference method of AOAC (2007). Watersoluble proteins were determined by Lowry method with bovine serum albumin (BSA) as standard. absorbance of water-soluble proteins measured using was spectrophotometer (Human X-ma 1200, China) at a wavelength of 760 nm (Waterborg and Matthews, 1994).

Identification of volatile compounds by GC-MS

The volatile compounds of the samples were analyzed on a headspace solidphase micro-extraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) system (GC 7890A; MS 5975C, Agilent Technologies, Santa Clara, CA), based on Laohakunjit et al. (2014). Algal sample was placed into a 22-mL vial and heated at 60°C for 10 min in a GCheating block for headspace analysis. Volatile compounds were absorbed onto an SPME fiber (50/30 DVB/CarboxenTM/PDMS μm StableFlexTM; Supelco, Bellefonte, PA) for 20 min. After equilibrium, the SPME fiber was desorbed into the injector port at 250°C for 20 min, and the injector was operated in splitless mode. Helium was used as the carrier gas at a constant velocity of 1.0 mL/min. Volatile compounds were separated using a DB-WAX capillary column (30 m x 250 µm x 0.25 µm; JandW Scientific Inc., Folsom, CA). The oven temperature program was set up as follows: initial temperature of 55°C; increased to 180°C at 5°C/min; increased to 200°C at 8°C/min; and held at 200°C for 10 min. Volatile compounds were detected using MSD (scan range of m/z 29-550) at 230°C. MS results were then recorded using electron impact at 70eV. The total ion count (TIC) was yielded and used for data identification and quantification (area). The TIC was compared to the spectral component database known in the GC-MS library (NIST-14), the identified flavors were then described using a flavor information database at www.flavornet.org and some published papers.

Experimental design for optimization using a response surface methodology (RSM)

The highest yield obtained in the preliminary at bromelain to substrate ratio of 50% and 3 h hydrolysis time suggested these values to be used as center points of the new experimental design. The ratio of bromelain to substrate (X₁) of 21.72%; 30%; 50%; 70%; 78.28% and hydrolysis time (X₂) of 0.17 h; 1 h; 3 h; 5 h; 5.83 h were used for a central composite design

with five center points and the final combinations of the factors are

described in Table 1.

Table 1: Central composite design matrix with calculated values of dependent variable.

Run	Coded variables		Independent va	Dependent variable	
	Ratio of bromelain	Hydroly	Ratio of bromelain	Hydrolysis	Yield (%)±SD*
	enzyme to substrate	sis Time	enzyme to	Time (h)	
	(% w/w)	(h)	substrate (% w/w)		
1	$\sqrt{2}$	0	78.28	3.00	0.3290±0,0044
2	0	0	50.00	3.00	$0.2662\pm0,0234$
3	-1	-1	30.00	1.00	$0.2584\pm0,0131$
4	1	1	70.00	5.00	$0.2756\pm0,0271$
5	$-\sqrt{2}$	0	21.72	3.00	$0.2133\pm0,0031$
6	-1	1	30.00	5.00	$0.2073\pm0,0088$
7	0	-√2	50.00	0.17	$0.2165\pm0,0088$
8	1	-1	70.00	1.00	$0.2711\pm0,0318$
9	0	0	50.00	3.00	0.2767 ± 0.0159
10	0	$\sqrt{2}$	50.00	5.83	$0.2093\pm0,0189$
11	0	0	50.00	3.00	$0.2753\pm0,0118$
12	0	0	50.00	3.00	$0.2644\pm0,0133$
13	0	0	50.00	3.00	$0.2663\pm0,0200$

^{*}SD was obtained from three replicates of each running

Process optimization

The value of sequential model sum of squares (SMSS), *lack of fit*, R-square, and adjusted-R square were determined to obtain the most representative optimization model. The valid model was considered p-value of lower than 0.05, not significant lack of fit (*p*>0.05), high R-square and adjusted R-square

(Montgomery, 2005). The optimal solution was investigated through a numerical optimization by setting up the goal of independent and dependent variables (minimization/maximization/on target), followed by determining the degree of importance (1-5) showed in the Table 2.

Table 2: Optimum values of process parameters and responses.

Variable	Constraints				
	Goal	Lower limit	Upper limit	Importance	
Ratio of bromelain enzyme to substrate (% w/w)	In range	21.72	78.28	3	
Hydrolysis Time (h)	In range	0.17	5.83	3	
Yield (%)	Maximize	0.2073	0.329	5	

Statistical analysis

Regression analysis and analysis of variance (ANOVA) were performed to investigate the effect of ratio of bromelain to substrate and hydrolysis time on the water-soluble protein yield. This work was performed using

Design-Expert software (Version 7.1.6. Stat-Ease Inc., Minneapolis, MN, USA) with a confidence level of 95%.

Optimization model verification

The verification of the optimal solution was carried out in laboratory

benchwork in triplicate. The differences between predicted and verified yields values would be considered to validate the model developed *in silico*. A difference of less than 5% would render the model valid (Wu *et al.*, 2008). The differences were calculated following the equation:

% Difference =
$$\frac{|\text{Observed yield - Estimated yield}|}{|\text{Observed yield}|} \times 100$$
 (2)

Results

Analysis of surface response methods in the production of sea grape protein hydrolysate

According to the regression analysis (Table 3), a quadratic or second order polynomial model was the most suitable for this study. The regressions coefficients when fitting the results to a quadradic model gave R-square and adjusted R-square of 0.9067 (90.67%) and 0.84 (84%) respectively, which implied that the factors considered (E/S

and time) affected the reaction in about 90.67% and only 9.33% of the results were dependent on the other factors that did not consider. PRESS (predicted residual error sum of squares) value for this model (0.0092) was the lowest among all models. The analysis of variance (Table 4) also supported the quadratic model as the F (13.60) and p-value (0.0017) were significant; the p-value represented that the noise in this experiment was very low (0.17%).

Table 3: Regression coefficients of the quadratic/second-order polynomial model for the dependent variable

variab	le				
Model	SD	R-Squared	Adjusted R- Squared	Predicted R Squared	PRESS
Linear	0.0266	0.5268	0.4321	0.0292	0.0145
2FI	0.0265	0.5784	0.4379	0.0199	0.0147
Quadratic	0.0141	0.9067	0.8400	0.3853	0.0092
Cubic	0.0087	0.9748	0.9395	-0.0629	0.0159

Table 4: Analysis of variance (ANOVA) of second-order polynomial model.

Sources	Estimated coefficient	F	P
Model	-	13.60	0.0017*
Constant	0.27	-	-
X_1	0.031	37.49	0.0005*
\mathbf{X}_2	-0.0071	2.02	0.1982
$X_1 * X_2$	0.014	3.87	0.0897
X_1^2	0.0035	0.42	0.5395
$X_2^{\frac{1}{2}}$	-0.0260	22.98	0.0020*

^{*}Significance (p<0,01); Adeq Precision 12,916.

The Adeq precision was 12.916 (>4), which could indicate high reliability and accuracy of the study. The

quadratic polynomial model obtained from the experimental design was below:

Yield (%) =
$$0.27+0.031X_1-0.0071X_2+0.014X_1X_2+0.0035X_1^2-0.026X_2^2$$
 (3)

The above quadratic model represented that enzyme to substrate ratio (X_1) and hydrolysis time (X_2) had positive and negative correlation to the watersoluble protein yield, respectively. The p-value of ratio of enzyme to substrate and reaction time was 0.0005 and 0.1982, respectively. This indicated that ratio of enzyme to substrate

significantly affected the water-soluble protein yield, while reaction time did not significantly affect the yield. The surface plot (Fig. 1) showed a correlation to the above developed model, where the surface plot showed a steeper trend of enzyme to substrate ratio than hydrolysis time.

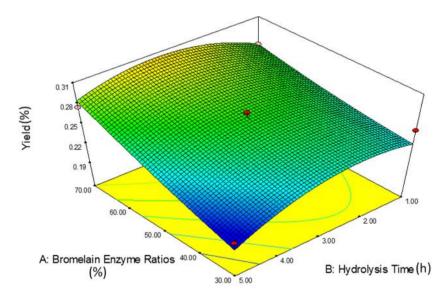


Figure 1: Response surfaces from the interaction between ration of enzyme to substrate and hydrolysis time.

Verification of the quadratic model

The optimal solution in the present study was obtained from a combination of E/S ratio and hydrolysis time of 78.28% and 3.49 h, respectively. The yield obtained between predicted and observed values are 0.3215 and 0.3104±0.0141, respectively with a difference of 3.58% (below 5%). This finding represented that we have

developed a valid quadratic model predicting the yield of protein hydrolysate in the present study, with desirability value of 0.9380.

Analysis of volatile compounds

The GC-MS data in Table 5 represented that the volatile compounds detected in both dried algal sample and hydrolysate were grouped to hydrocarbon,

aldehyde, alcohols, ketone, and fatty acids. Fifty-four compounds were detected in dried alga, while 58 compounds detected in hydrolysate. Most detected compounds in dried algal

samples were detected in hydrolysate, this information represented that hydrolysis carried out in the present study could release the aromatic compounds from dried algal sample.

Table 5: Volatile Compounds obtained between air dried sea grape and sea grape protein hydrolysate.

Notatile Compounds	A	rea*	Flavor description**	
-	Air-dried sea grape	Sea grape protein hydrolysates		
Hydrocarbons (aliphatic, cyclic,	aromatic)			
5-Octadecene, (E)-	\checkmark	\checkmark	Mild hydrocarbon	
Cyclododecane	\checkmark	\checkmark	Paraffin	
Pentadecane	\checkmark	\checkmark	Mild odor	
Octadecane, 2-methyl-	\checkmark	\checkmark	Fuel-like	
Naphthalene	\checkmark	\checkmark	Coal tar	
Pentadecane, 4-methyl-	\checkmark	\checkmark	Mild odor	
Tetradecane, 4-methyl-	\checkmark	\checkmark	Gasoline-like	
Benzene Pentadecane, 3-methyl-	\checkmark	\checkmark	Mild odor	
Heptadecane	\checkmark	\checkmark	Oily, Fuel-like	
1-Decene, 2,4-dimethyl-	\checkmark	\checkmark	Gasoline-like	
Pentadecane, 2,6,10-trimethyl-	\checkmark	\checkmark	Mild odor	
1-Pentadecene	\checkmark	\checkmark	Mild	
1-Heptadecene	\checkmark	\checkmark	Alkenes	
Tridecane, 6-propyl-	\checkmark	\checkmark	Gasoline-like	
Hexadecane, 1,1'-oxybis-	√	√	Gasoline-like	
8-Heptadecene	√	√	Fatty	
Pyridine, 4-ethyl-2,6-dimethyl-	√	√	Meaty, roasted	
Octadecane, 4-methyl-	√	√	Fuel-like	
Dodecane, 2,6,11-trimethyl-	✓	√	Coconut, fatty, waxy	
Eicosane	√	√	Waxy	
Heptadecane, 3-methyl-	√	√	Oily, Fuel-like	
2-Tetradecene, (E)-	√	√	-	
Caryophyllene oxide	√	√	Spicy, woody, terpenic	
Tetra Triacontyl	√	√	Rancid	
pentafluoropropionate	V	V	14411010	
Lilial	\checkmark	\checkmark	Floral, muguet, watery,	
			green, powdery, cumin	
2-Ethylhexyl salicylate	\checkmark	\checkmark	Mild, orchid, sweet, balsam	
Isopropyl myristate	\checkmark	\checkmark	Oily, fatty	
9-Methylheptadecane	\checkmark	\checkmark	Odorless	
Naphthalene, 2-methyl-1-propyl-	\checkmark	\checkmark	Sweet, floral, woody	
1-Acetyl-4,6,8-trimethylazulene	\checkmark	\checkmark	Green, spicy, sweet	
Versalide	\checkmark	\checkmark	Sweet, musk	
Diisobutyl phthalate	\checkmark	\checkmark	Ester odor	
Trichloromethane	\checkmark	-	Ether-like	
Hexadecane, 2,6,10,14-tetramethyl-	\checkmark	-	Gasoline-like	

Table 5 continued;				
Cyclopropane, 1-heptyl-2-	\checkmark	-	Sweet	
methyl-				
2-Methyloctacosane	\checkmark	-	Fatty, oily	
β-Myrcene	-	\checkmark	Peppery, balsam	
trans-β-Ocimene	-	\checkmark	Oily, sweet	
cis-β-Ocimene	-	\checkmark	Oily, sweet	
Neo-allo-ocimene	-	\checkmark	Sweet, floral, nut, skin, peppery, herbal, tropical	
Alloocimene	-	\checkmark	Sweet, floral, nut, skin, peppery, herbal, tropical	
β-Ocimene	-	\checkmark	Oily, sweet	
Terpinolene	-	\checkmark	Oily	
(+)-4-Carene	-	\checkmark	Sweet, pungent	
Allo-neo-Ocimene	-	\checkmark	Sweet, floral, nut, skin, peppery, herbal, tropical	
Benzene, 1,3-bis(1,1-dimethylethyl)-	-	√	Cooked beef	
Aldehyde				
Nonanal	\checkmark	\checkmark	Geranium, plastic, marine	
Hexanal	\checkmark	\checkmark	Fishy, grassy, leafy, green	
2-Octenal, (E)-	\checkmark	\checkmark	Fishy, oily, Green	
Heptanal	\checkmark	\checkmark	Burnt fat, citrus, rancid	
2,4-Decadienal, (E,E)-	\checkmark	\checkmark	Fishy, beef, potatocips	
2-Heptadecene	\checkmark	\checkmark	Seaweed-like	
Octanal, 2-(phenylmethylene)-	\checkmark	\checkmark	Grassy, leafy, green, fatty	
Benzaldehyde	\checkmark	\checkmark	Sweet, oily, nutty, woody	
Alcohol				
Ethanol, 2-phenoxy-	\checkmark	\checkmark	Mild, rose, balsam, cinnamyl	
2,4-Ditert-butylphenol	\checkmark	\checkmark	Fermented sausage	
Phenol, 4-(1-methylpropyl)-	\checkmark	\checkmark	Phenol-like	
Phenol	\checkmark	\checkmark	Phenol-like	
2,4-Ditert-butylphenol	\checkmark	-	Fermented sausage	
1-Octanol, 2-butyl-	\checkmark	-	Waxy, green, orange, aldehydic, rose, mushroom	
2,4-Ditert-butylphenol	\checkmark	-	Fermented sausage	
Ketone				
trans-β-Ionone	\checkmark	\checkmark	Floral, woody, violet	
2-Hexanone, 3,3-dimethyl-	-	\checkmark	Acetone like odor	
2,3-Octanedione	-	√	Coffee, brown	
Fatty acid			T	
Octanoic acid √ (detected)	√	√	Fatty, rancid, vegetable	

 $[\]sqrt{\text{(detected)}}$

Some compounds detected in algal sample such as trichloro methane, hexadecane, 2,6,10,14-tetramethyl-

cyclopropane, cyclopropane, 1-heptyl-2-methyl-,2-methyloctacosane, 2,4-ditert-butylphenol, 1-octanol, 2-butyl-,

^{- (}not detected)

^{*} GC-MS results were obtained using DB-WAX

^{**} Odor descriptions were cited from www.flavornet.org and recent reports.

2,4-di-tert-butylphenol were not detected in the sea grape protein hydrolysate. This information represented that hydrolysis, which applied high temperature caused the volatile compound loss. On the other compounds such hand, 12 myrcene, trans-β-ocimene, cis-B-Ocimene. neo-allo-ocimene, Alloocimene, β-ocimene, terpinolene, (+)-4-carene, allo-neo-ocimene, benzene, 1,3-bis(1,1-dimethylethyl)-, 2-Hexanone, 3,3-dimethyl-, and octanedione detected were in hydrolysate

Discussion

Our study has developed a second order polynomial (quadratic model) to predict the effect of ratio of bromelain enzyme to substrate and reaction time on the water-soluble protein vield. statistical parameters such as R-square, adjusted R-square, lack of fit test, model p-value, PRESS (predictive sum of squares) and Adeq precision, determined in the response fitted second order polynomial model in the present study. According to the regression analysis and analysis of variance in Table 3 and 4, all parameters required to fit the developed model meet the requirements. Therefore, the model chosen was reliable.

The optimal solution criterion suggested by Montgomery (2005) was solution with a high desirability value, where it is the higher of the desirability value, the more accurate of the optimal solution. We verified the model used to

predict the optimal solution by performing an experimental comparing the difference level between predicted and validated optimal solution. The verification showed that the difference level was less than 5%. where this value suggested that models established were accurate, and could elucidate the effect of ratio bromelain enzyme to substrate and reaction time on the water-soluble protein yield. However, a prior study suggests 10% in the level of difference between predicted and validated values (Mabazza *et al.*, 2020).

Our further analysis on the production scenario of water-soluble from Caulerpa protein racemosa showed that the ratio of enzyme to substrate had positive correlation to the protein yield, while reaction time had the negative correlation. This model could be either elucidated by the surface plot presented (Fig. 1), which showed that the more the ratio of enzyme to substrate, the higher yield of water-soluble protein obtained. However, the reaction time factor showed a stationary phase at around three hours, followed by declining trend of water-soluble protein yield after that. The presence of stationary peak over the enzymatic reaction was in line with three previous studies by Sonklin et al. (2011), Laohakunjit et al. (2014) and Utami et al. (2019) which also show that the steeper curves in the variation of the bromelain enzyme ratios indicate that the higher of the bromelain enzyme ratios can increase the yield produced,

but at a certain time the enzyme activity will reach a stationary phase and then it will decrease slowly. Hence, enzymatic reaction typically raises a stationary phase over the reaction period.

The trend of enzymatic reaction by bromelain enzyme either increasing or decreasing in the present study could be referred to some possible mechanisms. The initial phase is a significant phase in the enzymatic reaction, where in this phase enzymatic reaction rapidly occurs (Haard, 2001; Himonides et al., 2011). During an initial phase, the amount of substrate binding to the active site of the enzymes is still abundant, which facilitates the formation enzyme-substrate complex (Sonklin et al., 2011). According to Ovissipour et al. (2012), the enzymatic reactions convert water-insoluble proteins into water-soluble nitrogen compounds; therefore, the water-soluble protein content increases during hydrolysis.

The declining phase could be caused by several factors. Berg et al. (2002) stated that the declining is typically enzymatic reaction when the enzyme saturated by the substrate. Kanu et al. (2009) and Murray et al. (2009) showed that the stationary phase occurs due to lower substrate availability, which cause the impossibilities to form E/S complex, so that water-soluble protein could not be produced. A prior study challenges elucidated the of hydrolysing protein from the whole algal tissue was the presence of some polysaccharides, which might inhibit

the enzymatic reaction (Laohakunjit *et al.*, 2014).

Analysis of volatile compounds in air-dried *Caulerpa racemosa* and protein hydrolysates have been carried out (Table 5). Hydrocarbon was the dominant volatile compound detected by GC-MS in the present study, which were obtained from the decarboxylation reaction and the carbon chain bonding process of fatty acids, as well as thermal oxidation of unsaturated fatty acids (Chung *et al.*, 2002; Linder and Ackman, 2002; Liu *et al.*, 2009).

Aldehyde was the second abundantly detected compound in both whole sea hydrolysate. grape and Octanal. benzaldehyde, nonanal and hexanal are the indigenous aldehyde compound in some algae with fishy or crab flavor (Qi et al., 2017). These compounds are products of biosynthesis and bioregeneration of fatty acids in the algae (Boonprap et al., 2006). However, these compounds could be either affected by the enzymatic reaction during the airdrying because the slow water evaporation during air drying could trigger the enzymatic reaction (Stévant et al., 2018). According to Caprino et al. (2008), hexanal, heptanal, octanal, and nonanal are produced from oleic acid and linoleic acid as precursors, where during the hydrolysis the lipid release occurs due to the degradation of cell wall of algae due to enzymatic reaction. This is presumably due to the lipid content in Caulerpa racemosa used in our study. Kajiwara et al. (1993)that showed green algae

relatively have more aldehyde volatile compounds than brown and red algae; this information suggested that *Caulerpa racemosa* could be a potential source of sea food flavouring agents.

The group of detected alcohol compounds released bv was decomposition of secondary hydroperoxides from fatty acids (Girard and Durance, 2000). According to Shalit et al. (2001), aldehyde was potential precursor of alcohols which the catalysed by alcohol dehydrogenase. Ketones formed in sea grape protein hydrolysate are expected as products of thermal oxidation and decomposition of fatty acids, especially unsaturated fatty acids (Liu et al., 2009).

Some detected volatile compounds were known to be source of sweet flavour such as benzaldehyde, myrcene and ocimene. According to Kobayashi and Odake (2019), these compounds are products of Maillard reaction which caused by the presence polysaccharide and amino acid in the algal tissue. The use of high temperature in producing protein hydrolysate in study might our stimulate the Maillard reaction (Trevisan et al., 2016). Indeed, Bak et al. (2018) reported that benzaldehyde is the significant marker of Maillard reaction during hydrolysis by papain concentration enzyme, which the quantitively increased with the increasing of E/S ratio during the reaction.

On one hand hydrolysis triggered the presence of new compounds. On the other hand, there were some volatile compounds lost during protein hydrolysis. According to Henrickson (2005), the low boiling point of some volatile compounds increase their volatility, therefore the application of heating during hydrolysis causes the loss of volatile compounds.

The volatile compounds investigated in the present study were also found in other seafood or marine organisms, such as pentadecane and octadecane which are found in coral species of Patinopecten vessoensis (Chung et al., 2002), silver carp fish (Liu et al., 2009); ketones in various types of crustaceans (Morita et al., 2003); benzaldehyde, octanoic acid. pyridine, phenols generating shrimp flavour (Morita et al..2003; Yu and Chen, Laohakunjit et al., 2014; Mouritsen et al., 2019).

To sum up, the process optimization by using the response surface methodology has robustly developed a valid quadratic model, which could elucidate the effect of ratio of enzyme to substrate and reaction time on the yield of watersoluble protein from sea grape. The statistical analysis showed that the ratio of enzyme to substrate significantly affected the protein yield, but reaction time did not significantly affect the protein yield. The optimal solution in the present study was achieved by the combination of enzyme to substrate ratio of 78.28% and hydrolysis time of 3.49 h, which yielded 0.3215% (w/w) of water-soluble protein. The present study showed that hydrolysis with bromelain could release volatile compounds from air-dried seaweed and formation stimulate the of compounds as the result of enzymatic degradation. Considering the prior studies on sea food flavor, sea grapes protein hydrolysate produced using bromelain as shown in this study, have great potential to be used as sea food flavoring agent.

Acknowledgement

Thanks to Faculty of Fisheries and Marine Universitas Airlangga for the Grant Annual Research through Rencana Kerja Anggaran **Tahunan** (RKAT) for Research Scheme 2018. Thanks to PT. Bromelain Enzyme, Lampung Province, Indonesia donating bromelain enzyme to perform the present study. Thanks to Dr. Ganden Supriyanto (orcid:0000-0002-8881-4871), an analytical Chemist of Universitas Airlangga for assisting us in selecting the best water-soluble protein method. Thanks to my Muslim community of Dzemat Kevser Tulln, Austria for donating a new laptop, which could be used to finalize this manuscript.

References

- AOAC (Official Methods of Analysis), 2007. 18th Edition, Association of Official Analytical chemists, Gaithersburg.
- Arshad, Z.I.M., Amid, A., Yusof, F., Jaswir, I., Ahmad, K. and Loke,

- **S.P., 2014.** Bromelain: An overview of industrial application and purification strategies. *Applied Microbiology and Biotechnology*, 98(17), 7283–7297. DOI:10.1007/s00253-014-5889-y
- Bak, K., Petersen, M., Lametsch, R., Hansen, E. and Ruiz-Carrascal, J., 2018. Development of volatile compounds during hydrolysis of porcine hemoglobin with papain. *Molecules*, 23(2), 357. DOI:10.3390/molecules23020357
- Berg, J.M., Tymoczko, J.L. and Stryer, L., 2002. Biochemistry, 5th ed. USA. WH Freeman.
- Boonprab, K., Matsui, K., Akakabe, Y., Yoshida, M., Yotsukura, N., Chirapart, A. and Kajiwara, T., 2006, Formation of aldehyde flavor (n-hexanal, 3Z-nonenal and 2E-nonenal) in the brown alga, Laminaria angustata, Journal of Applied Phycology, 18(3), 409-412. DOI:10.1007/s10811-006-9038-6
- Caprino, F., Moretti, V.M., Bellagamba, F., Turchini, G.M., Busetto, M.L., Giani, I. and Pazzaglia, M., 2008. Fatty acid composition and volatile compounds of caviar from farmed white sturgeon (Acipenser transmontanus). Analytica Chimica Acta, 617(1–2), 139–147.

DOI:10.1016/j.aca.2008.02.005

Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R. and Jyothirmayi, T., 2012. Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities

- and applications: A review. *Food Chemistry*, 135(**4**), 3020–3038. DOI:10.1016/j.foodchem.2012.06.10
- Chung, H.Y., Yung, I.K.S., Ma, W.C.J. and Kim, J.S., 2002.

 Analysis of volatile components in frozen and dried scallops (*Patinopecten yessoensis*) by gas chromatography/mass spectrometry. Food Research International, 35(1), 43-53. DOI:10.1016/S0963-9969(01)00107-7
- Dang, Y., Gao, X., Ma, F. and Wu, X., 2015. Comparison of umami taste peptides in water-soluble extractions of Jinhua and *Parma hams*, *LWT Food Science and Technology* 60(2), 1179-1186. DOI:10.1016/j.lwt.2014.09.014
- de Lencastre Novaes, L.C., Jozala, A.F., Lopes, A.M., de Carvalho SantosEbinuma, V., Mazzola, P.G., Pessoa Junior, A., 2016. Stability, purification, and applications of bromelain: A review". *Biotechnology Progress*, 32 (1) 5–13. DOI:10.1002/btpr.2190
- Fleurence, J., 2016. Seaweed as food, in Joël Fleurence, I.L. (Ed.), Seaweed in Health and Disease Prevention, Academic Press, Waltham, pp. 149–167.
- Girard, B. and Durance, T., 2000. Headspace volatiles of sockeye and pink salmon as affected by retort process. *Journal of Food Science*, 65(1), 34–39. DOI:10.1111/j.1365-2621.2000.tb15952.x
- Haard, N.F., 2001. Enzymic

- modification of proteins in food systems, in Sikorski, Z.E. (Ed.), *Chemical and Functional Properties of Food Proteins*, CRC Press, Boca Raton, pp. 155–190.
- **Henrickson, C., 2005.** Chemistry, Cliffs Notes. hoboken, new jersey: wiley publishing, Inc.
- Himonides, A.T., Taylor, A.K. and Morris, A.J., 2011. A study of the enzymatic hydrolysis of fish frames using model systems. *Food and Nutrition Sciences*, 2(6), 575-585. DOI:10.4236/fns.2011.26081
- Horstmann, U., 1983. Cultivation of the green alga, *Caulerpa racemosa*, in tropical waters and some aspects of its physiological ecology, *Aquaculture*, 32(3-4), 361-371. DOI:10.1016/0044-8486(83)90233-8
- Huang, W., Li, Z., Niu, H., Li, D. and Zhang, J., 2008. Optimization of operating parameters for supercritical carbon dioxide extraction of lycopene by response surface methodology. *Journal of Food Engineering*, 89(3), 298-302. DOI:10.1016/j.jfoodeng.2008.05.006
- Kajiwara, T., Matsui, K., Hatanaka, A., Tomoi, T., Fujimura, T. and Kawai, T., 1993. Distribution of an enzyme system producing seaweed flavor: conversion of fatty acids to long-chain aldehydes in seaweeds. *Journal of Applied Phycology*, 5(2), 225-230. DOI:10.1007/BF00004022
- Kanu, P.J., Kanu, J.B., Sandy, E.H.,Kandeh, J.B.A., Mornya, P.M.P.and Huiming, Z., 2009.Optimization of enzymatichydrolysis

of defatted sesame flour by different proteases and their effect on the functional properties of the resulting protein hydrolysate. *American Journal of Food Technology*, 4(6), 226-240.

DOI:10.3923/ajft.2009.226.240

- Kobayashi, F. and Odake, S., 2019. Application of a two-stage system with pressurized carbon dioxide microbubbles for inactivating enzymes and microorganisms in unpasteurized sake and unfiltered beer. Alcoholic Beverages: Volume 7: The Science of Beverages.
- Laohakunjit, N., Selamassakul, O. and Kerdchoechuen, O., 2014. Seafood-like flavor obtained from the enzymatic hydrolysis of the protein by-products of seaweed (*Gracilaria* sp.). Food Chemistry, 158, 162-170. DOI:10.1016/j.foodchem.2014.02.10
- Linder, M. and Ackman, R.G., 2002.

 Volatile compounds recovered by solid-phase microextraction from fresh adductor muscle and total lipids of sea scallop (Placopecten magellanicus) from georges bank (Nova Scotia). *Journal of Food Science*, 67(6), 2032-2037.

 DOI:10.1111/j.1365-2621.2002.tb09496.x
- Liu, J.K., Zhao, S.M., Xiong, S.B. and Zhang, S.H., 2009. Influence of recooking on volatile and non-volatile compounds found in silver carp *Hypophthalmichthys molitrix*. *Fisheries Science*, 75(4), 1067-1075.

- DOI:10.1007/s12562-009-0116-y
- Ma'ruf, W.F., Ibrahim, R., Dewi, E.N., Susanto, E. and Amalia, U., 2013. Caulerpa racemosa and Gracilaria verrucosa Profile as Edible Foods. *Saintek Perikanan*, 9(1), 68-74.
- Mabazza, K.A.A., Requiso, P.J., Alfafara, C.G., Nayve Jr, F.R.P. and Ventura, J.R.S., 2020. Steam Explosion and Sequential Steam Explosion–Dilute Acid Pretreatment Optimization of Banana Pseudostem for Polyhydroxybutyrate (PHB) Production. *Philippine Journal of Science*, 149(2), 285-297.
- **Montgomery, D.C., 2005.** Design and analysis of experiments. John Wiley and Sons, New York.
- Morita, K., Kubota, K. and Aishima, T., 2003. Comparison of aroma characteristics of 16 fish species by sensory evaluation and gas chromatographic analysis. *Journal of the Science of Food and Agriculture*, 83(4), 289-297. DOI:10.1002/jsfa.1311
- Mouritsen, O.G., Duelund, L., Petersen, M.A., Hartmann, A.L. and Frøst, M.B., 2019. Umami taste, free amino acid composition, and volatile compounds of brown seaweeds. *Journal of Applied Phycology*, 31(2), 1213-1232. DOI:10.1007/s10811-018-1632-x
- Murray, R., Kennelly, P., Bender, D., Rodwell, V., Botham, K., and Weil, P. A., 2009. Harper's Illustrated Biochemistry 28th ed. USA: Mcgraw-hill.

- Nasri, M., 2017. Protein Hydrolysates and Biopeptides: Production, Biological Activities, and Applications in Foods and Health Benefits. A Review". Advances in Food and Nutrition Research, 81, 109-159.
 - DOI:10.1016/bs.afnr.2016.10.003
- Nuraini, R., 2006. Percobaan Berbagai Macam Metode Budidaya Latoh (*Caulerpa racemosa*) Sebagai Upaya Menunjang Kontinuitas Produksi. *Indonesian Journal of Marine Sciences*, 11, 101-105. DOI:10.14710/ik.ijms.11.2.101-105
- Ovissipour, M., Safari. **R..** Motamedzadegan, A. and Shabanpour, B., 2012. Chemical Biochemical Hydrolysis of Persian Sturgeon (Acipenser persicus) Visceral Protein. Food and Bioprocess Technology, 5(2), 460-465. DOI:10.1007/s11947-009-0284-x
- Pereira, L., 2011. A review of the nutrient composition of selected edible seaweeds. In Seaweed:

 Ecology, Nutrient Composition and Medicinal Uses, pp. 15-47.

 USA:Nova Science Publishers, Inc
- Qi, H., Xu, Z., Li, Y. bo, Ji, X. lin, Dong, X. fang and Yu, C., 2017. Seafood flavorings characterization as prepared from the enzymatic hydrolysis of Undaria pinnatifida sporophyll by-product. *International Journal of Food Properties*, 20(12), 2867-2876.
 - DOI:10.1080/10942912.2016.12563 02

- Rój, E., Dobrzyńska-Inger, A., Dębczak, A., Kostrzewa, D. and Stępnik, K., 2015. Algae Extract Production Methods and Process Optimization. *Marine Algae Extracts: Processes, Products, and Applications*, pp. 101-120.
- Sarkar, S., Ahmed, M., Mozumder, N.H.M.R. and Saeid, A., 2017. Isolation and characterization of bromelain enzyme from pineapple and its utilization as anti-browning agent. *Process Engineering Journal*, 1, 52-58.
- Shalit, M., Katzir, N., Tadmor, Y., Larkov, O., Burger, Y., Shalekhet, F., Edelstein, M., 2001. Acetyl-CoA: alcohol acetyltransferase activity and aroma formation in ripening melon fruits. *Journal of Agricultural and Food Chemistry*, 49(2), 794–799.
 - DOI:10.1021/jf001075p
- Sonklin, C., Laohakunjit, N. and Kerdchoechuen, O., 2011. Physicochemical and flavor characteristics of flavoring agent from mungbean protein hydrolyzed by bromelain. **Journal** Agricultural and Food Chemistry, 8475-8483. 59(**15**), DOI:10.1021/jf202006a
- Stévant, P., Indergård, E., Ólafsdóttir, A., Marfaing, H., W.E., Larssen, Fleurence, J., Roleda, M.Y., 2018. Effects of drying on the nutrient content and physico-chemical and sensory characteristics of the edible kelp Saccharina latissima. Journal of

Applied Phycology, 30(**4**), 2587-2599. DOI:10.1007/s10811-018-1451-0

- Trevisan, A. J. B., de Almeida Lima, D., Sampaio, G. R., Soares, R. A. M., Markowicz Bastos, D. H., 2016. Influence of home cooking conditions on Maillard reaction products in beef. *Food Chemistry*, 196, 161–169. DOI:10.1016/j.foodchem.2015.09.00
- Utami, T., Kusuma, E.N., Satiti, R., Rahayu, E.S. and Cahyanto, M.N., 2019. Hydrolyses of meat and soybean proteins using crude bromelain to produce halal peptone as a complex nitrogen source for the growth of lactic acid bacteria. *International Food Research*

Journal, 26(1).

- Waterborg, J.H. and Matthews, H.R., 1994. The Lowry method for protein quantitation. *Methods in Molecular Biology (Clifton, N.J.)*.
- Wu, M., Ding, H., Wang, S. and Xu, S., 2008. Optimizing conditions for the purification of linoleic acid from sunflower oil by urea complex fractionation. *JAOCS*, *Journal of the American Oil Chemists' Society*, 85(7), 677. DOI:10.1007/s11746-008-1245-7
- Yu, H.Z. and Chen, S.S., 2010. Identification of characteristic aroma-active compounds in steamed mangrove crab (*Scylla serrata*). Food Research International, 43(8), 2081-2086.

DOI:10.1016/j.foodres.2010.07.009