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## Research Article

# Optimization of production of enzymatic protein hydrolysate-based flavor from sea grape (*Caulerpa racemosa*)

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## 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 50ºC, followed by heating at 95ºC 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

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## Introduction

*Caulerpa racemosa* (colloquially 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 al.*, 2013). Interestingly, those 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 is 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 via either chemical or enzymatic hydrolysis, although enzymatic hydrolysis is relatively preferable because it is easily controlled, efficient, reproducible, eco-friendly, 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 food-grade 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

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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 their 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% $Na_2CO_3$ in 0.1 N NaOH and 0.5% $CuSO_4$ 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 of water-soluble proteins were determined according to the following equation:

$$\text{Yield (\%w/w)}=\frac{\text{Protein level }\left(\frac{\text{ug}}{\text{mL}}\right)\text{x hydrolysate volume (mL)}}{\text{Seaweed weight (ug)}}\times 100 \qquad (1$$

*Chemical analysis*

Protein content of algal sample was determined according to reference method of AOAC (2007). Water-soluble proteins were determined by Lowry method with bovine serum albumin (BSA) as standard. The absorbance of water-soluble proteins was measured using a 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 solid-phase 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 GC–MS heating block for headspace analysis. Volatile compounds were absorbed onto an SPME fiber (50/30 µm DVB/Carboxen™/PDMS StableFlex™; 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 ($X_1$) of 21.72%; 30%; 50%; 70%; 78.28% and hydrolysis time ($X_2$) of 0.17 h; 1 h; 3 h; 5 h; 5.83 h were used for a central composite design

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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 variables | | Dependent variable |
|---|---|---|---|---|---|
| | Ratio of bromelain enzyme to substrate (% w/w) | Hydrolysis Time (h) | Ratio of bromelain enzyme to substrate (% w/w) | Hydrolysis Time (h) | Yield (%)±SD* |
| 1 | $\sqrt{2}$ | 0 | 78.28 | 3.00 | 0.3290±0,0044 |
| 2 | 0 | 0 | 50.00 | 3.00 | 0.2662±0,0234 |
| 3 | -1 | -1 | 30.00 | 1.00 | 0.2584±0,0131 |
| 4 | 1 | 1 | 70.00 | 5.00 | 0.2756±0,0271 |
| 5 | $-\sqrt{2}$ | 0 | 21.72 | 3.00 | 0.2133±0,0031 |
| 6 | -1 | 1 | 30.00 | 5.00 | 0.2073±0,0088 |
| 7 | 0 | $-\sqrt{2}$ | 50.00 | 0.17 | 0.2165±0,0088 |
| 8 | 1 | -1 | 70.00 | 1.00 | 0.2711±0,0318 |
| 9 | 0 | 0 | 50.00 | 3.00 | 0.2767±0,0159 |
| 10 | 0 | $\sqrt{2}$ | 50.00 | 5.83 | 0.2093±0,0189 |
| 11 | 0 | 0 | 50.00 | 3.00 | 0.2753±0,0118 |
| 12 | 0 | 0 | 50.00 | 3.00 | 0.2644±0,0133 |
| 13 | 0 | 0 | 50.00 | 3.00 | 0.2663±0,0200 |

*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

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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:

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

## 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**

| 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* |
| $X_2$ | -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 |
| $X_2^2$ | -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:

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

The above quadratic model represented that enzyme to substrate ratio ($X_1$) and hydrolysis time ($X_2$) had positive and negative correlation to the water-soluble 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.

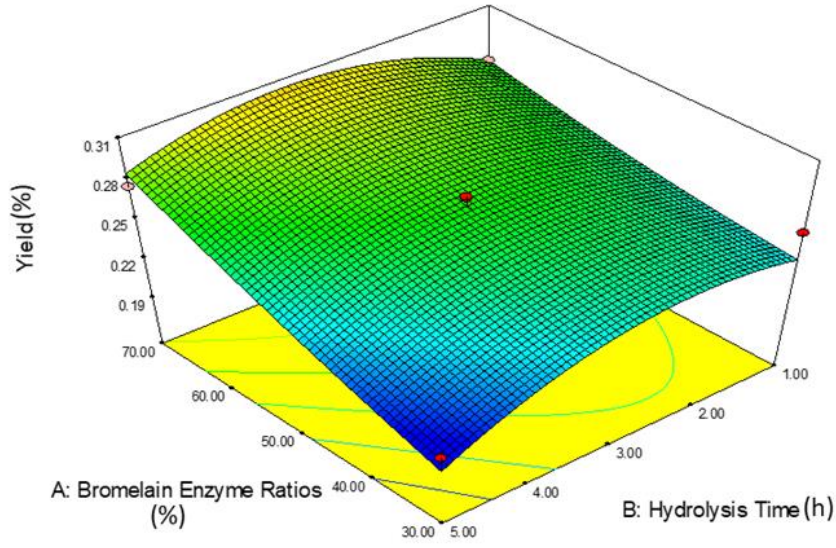

**Figure 1: Response surfaces from the interaction between ration of enzyme to substrate and hydrolysis time.**

### *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,

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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.**

| **Volatile Compounds** | **Area*** | | **Flavor description**** |
|---|---|---|---|
| | **Air-dried sea grape** | **Sea grape protein hydrolysates** | |
| **Hydrocarbons (aliphatic, cyclic, aromatic)** | | | |
| 5-Octadecene, (E)- | √ | √ | Mild hydrocarbon |
| Cyclododecane | √ | √ | Paraffin |
| Pentadecane | √ | √ | Mild odor |
| Octadecane, 2-methyl- | √ | √ | Fuel-like |
| Naphthalene | √ | √ | Coal tar |
| Pentadecane, 4-methyl- | √ | √ | Mild odor |
| Tetradecane, 4-methyl- | √ | √ | Gasoline-like |
| Benzene Pentadecane, 3-methyl- | √ | √ | Mild odor |
| Heptadecane | √ | √ | Oily, Fuel-like |
| 1-Decene, 2,4-dimethyl- | √ | √ | Gasoline-like |
| Pentadecane, 2,6,10-trimethyl- | √ | √ | Mild odor |
| 1-Pentadecene | √ | √ | Mild |
| 1-Heptadecene | √ | √ | Alkenes |
| Tridecane, 6-propyl- | √ | √ | 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 pentafluoropropionate | √ | √ | Rancid |
| Lilial | √ | √ | Floral, muguet, watery, green, powdery, cumin |
| 2-Ethylhexyl salicylate | √ | √ | Mild, orchid, sweet, balsam |
| Isopropyl myristate | √ | √ | Oily, fatty |
| 9-Methylheptadecane | √ | √ | Odorless |
| Naphthalene, 2-methyl-1-propyl- | √ | √ | Sweet, floral, woody |
| 1-Acetyl-4,6,8-trimethylazulene | √ | √ | Green, spicy, sweet |
| Versalide | √ | √ | Sweet, musk |
| Diisobutyl phthalate | √ | √ | Ester odor |
| Trichloromethane | √ | - | Ether-like |
| Hexadecane, 2,6,10,14-tetramethyl- | √ | - | Gasoline-like |

**Table 5 continued;**

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

√ (detected)
- (not detected)
* GC-MS results were obtained using DB-WAX
** Odor descriptions were cited from www.flavornet.org and recent reports.

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-di-tert-butylphenol, 1-octanol, 2-butyl-, 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 hand, 12 compounds such as β-myrcene, trans-β-ocimene, cis-β-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 2,3-octanedione were detected 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 yield. The 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 work 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 of 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 protein from *Caulerpa 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 then facilitates the formation of 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 elucidated the challenges 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 grape and hydrolysate. 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 bio-regeneration of fatty acids in the algae (Boonprap *et al*., 2006). However, these compounds could be either affected by the enzymatic reaction during the air-drying 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) showed that green algae

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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 was released by decomposition of secondary hydroperoxides from fatty acids (Girard and Durance, 2000). According to Shalit *et al.* (2001), aldehyde was potential precursor of alcohols which are catalysed by the 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 of polysaccharide and amino acid in the algal tissue. The use of high temperature in producing protein hydrolysate in our study might 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 enzyme, which the concentration 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 yessoensis* (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, 2010; 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 water-soluble 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)

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of water-soluble protein. The present study showed that hydrolysis with bromelain could release volatile compounds from air-dried seaweed and stimulate the formation of new 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.

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