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Contents

Activity of *Bacillus licheniformis* α-Amylase in Hydrolyzing Papua Sago Starch

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Abstract

α-Amylase (EC 3.2.1.1) catalyzes the hydrolysis of the α-1,4-glycosidic bonds present in oligosaccharides and polysaccharides. A hyperthermostable α-amylase from *Bacillus licheniformis* is widely used in industry. The objective of this research was to evaluate the hydrolytic activity of α-amylase from *B. licheniformis* using raw sago starch (*Metroxylon sagu* Rottbol) as a substrate. The optimum temperature and pH for this α-amylase were 85°C and 6.5, respectively. The activation energy (E_a) was 7.333 kJ/mol. The free energy (ΔG) was 50.843 kJ/mol, enthalpy (ΔH) was 4.855 kJ/mol, and entropy (ΔS) was -154.319 J/mol K. This α-amylase has a Michaelis-Menten constant (K_M) of 2.19% (w/v) and maximum velocity (V_{max}) of 625 units/mg, while the turnover number (k_{cat}) and specificity constant (k_{cat}/k_m) were 7,596.75/min and 3,468.34 mg/unit min, respectively. The glucose concentration in the hydrolysate was 27.568 µg/mL. The dextrose equivalent (DE) of the hydrolysate was 16.51%. HPLC analysis indicated that maltopentaose was the predominant species among other malto-oligosaccharides in the hydrolysate. Thus, Papua sago starch is potential to be used as an alternative cheap raw material in the maltodextrin industry.

Keywords: α-amylase; Bacillus licheniformis; dextrose equivalent; sago starch; maltodextrin

Introduction

Indonesia has about 1.128 million ha of sago palm growing areas (51.3% of the total of 2.201 million ha sago palm growing areas worldwide). The three countries that account for virtually all the remaining sago palm growing areas are Papua New Guinea (43.3%), Malaysia (1.5%), and Thailand (0.2%). Ninety percent of Indonesia's total area of sago palm is located in Papua

Province-Indonesia. The main species of sago palm found in Indonesia is *Metroxylon sagu* Rottbol which contained amylose (27%) and amylopectin (73%). Sago starch productivity in Indonesia is 25 tons/ha/year. The price of sago starch is about 0.16 US$/kg, which is less than that of cassava starch (0.20 US$/kg) and corn starch (0.31 US$/kg) (Dwiarti et al., 2007).

Starch is used as a raw material and/or as an additive in several commercial settings, including the food, pharmaceutical, textile and electronic industries. For instance, starch is used in the food industry as thickeners, gelling agents, bulking agents (fillers), water absorbents, and as energy sources in fermentation (Ohmes et al., 1998; Marchal et al., 1999; Nakuakuki, 2002; Richardson et al., 2002; Linden et al., 2003). In the starch processing industry, the liquefaction of starch is needed to produce maltodextrin (a mixture of monosaccharides, oligosaccharides, and short chain polysaccharides). The liquefaction process is dependent on endo-1,4-α-D-glucan glucanohydrolase (α-amylase, E.C. 3.2.1.1) enzyme. Alpha amylase catalyzes the hydrolysis of linear or branched starches, some 1,4-glycosidic oligosaccharides, and some 1,4-glycosidic polysaccharides in the "endo" conformation. Hydrolysis of starch by α-amylase is rapid, and the hydrolysate is in the α configuration (Nakuakuki, 2002; Linden et al., 2003; Hashida and Bisgaard-Frentzen, 2000).

The partial hydrolysis product of starch (maltodextrin) has a dextrose equivalent (DE) lower than 20 (Marchal et al, 1990). However, maltodextrins with different DEs have different functionalities and physicochemical properties. Maltodextrin could be used in food as a binder, as a bulking agent or as a flavor encapsulator. The unique properties of low DE maltodextrin, such as its fat-like texture in the mouth, have accelerated the development of fat replacements (Ohmes et al., (1998); Marchal et al., 1999). The need to reduce fat and calories in the diet has elevated the commercial importance of low DE maltodextrin within the food industry (Inglett dan Grisamore, 1991). Commercial maltodextrin can be produced from potato starch, corn starch, oat, rice, and tapioca (Artz and Hansen in Akoh and Swanson, 1994; Lucca and Tepper, 1994; Harkema in Roller and Jones, 1996; Roller and Jones, 1996).

Despite its commercial importance, the biochemical characteristics of hyperthermostable α-amylase as they relate to hydrolysis of sago starch are uncharacterized. In the present study we determined the optimum pH and temperature and its effects on dextrose equivalents (DEs), glucose production during hydrolysis, and malto-oligosaccharides composition in the hydrolysate. Kinetics and thermodynamics studies were also performed.

Materials and Methods

Materials

The materials used in this study included Papua sago starch (the source of the raw starch was identified as *Metroxylon sagu* Rottbol), α-amylase enzyme (Novozymes Bagsvaerd, Denmark) a thermostable bacterial α-amylase from *B. licheniformis*, PGO enzyme P7119 (*Aspergillus niger* glucose oxidase, horseradish peroxidase, *o*-dianisidine dihydrochloride (Sigma, Saint Louis, Missouri)), standard solutions for High Performance Liquid Chromatography (HPLC) (such as glucose (Degree of Polymerization 1 or DP1), DP2, DP3, DP4, DP5, DP6, DP7, DP8, DP9, DP10)

(Sigma, Saint Louis, Missouri) and a 1 mg/mL solution of standard glucose in benzoic acid 0.1% (w/v) (Sigma, Saint Louis, Missouri), Bovine Serum Albumin (BSA) (Sigma, Saint Louis, Missouri), and Folin-Ciocalteu reagent (Sigma, Saint Louis, Missouri). All chemicals used for preparing Nelson-Somogyi reagent, buffers and other reagents in this study were pro analytical or molecular biology grade, unless otherwise stated.

Preparation of substrate

Five hundred grams of raw sago starch was washed with 2 L of distilled water (washing five times with maceration and percolation); each wash lasted 10 min. After washing, the liquids were removed and the sago starch was oven-dried at 40°C for 24 h (Collado and Corke, 1997). Furthermore, 1 g of dried sago starch was dissolved in a glass beaker by adding 50 mL of 0.1 M acetate buffer (pH of 4.8). The solution mixture was then boiled. Subsequently, the volume was adjusted to 100 mL with the addition of boiling acetate buffer (i.e., final substrate concentration was 1%). The substrate solution was then cooled at room temperature and stored at 4^{o}C. For determination of optimum conditions and parameters for enzyme kinetics, several substrate concentrations were prepared. The same procedure was conducted to prepare the substrate solutions at pH 4.5, 5.0, and 5.5 by using 0.1 M acetate buffer. Preparation of a substrate solution with pH 6.0 was achieved with 0.1 M phosphate buffer, while 0.1 M Tris-maleic buffer was used to prepare both pH 6.5 and 7.0 substrate solutions.

Determination of protein content

Alpha amylase enzyme concentration was determined using the method of Lowry *et al.* (1951). Crystalline Bovine Serum Albumine (BSA) (Sigma) was used as the standard.

Enzymatic hydrolysis of sago starch

Hydrolysis was conducted by adding 0.1 mL of 1% sago starch solution and 0.1 mL of enzyme solution (α-amylase) in 1.5 mL Eppendorf tubes, which were then incubated in a Thermolyne block incubator at various temperatures (60-95^{o}C) for 20 min. The reaction was stopped by heating in boiling water for 20 min. The hydrolysate was cooled to room temperature. Little amount of hydrolysate (0.2 mL) was then used in the analysis of reducing sugar concentration by Nelson-Somogyi method (Somogyi, 1952), while the glucose concentration was determined using the glucose oxidase peroxidase assay described by Raabo and Terkildsen (Raabo and Terkildsen, 1960) with a kit from Sigma (St. Louis, MO).

Alpha amylase activity assay using the Nelson-Somogyi method

A hydrolysis procedure similar to the procedure described in section 2.4 was performed, except that the termination reaction of the Nelson-Somogyi method was performed by the addition of 0.3 mL of Somogyi-copper reagent to the reaction tube containing 0.3 mL hydrolysate (1:100). This mixture was then incubated in a boiling water bath for 20 min. Afterwards, the reaction mixture was cooled to room temperature and 0.3 mL of Nelson's arsenomolybdate reagent was added,

shaken by vortexing, and allowed to stand for 5 min. The reaction mixture was diluted by adding 2.1 mL of distilled water. Absorbance of the solution was read using a spectrophotometer at 700 nm and the reducing sugar content was determined. A calibration curve was prepared beforehand, with glucose concentrations ranging from 5–40 µg/mL. Alpha amylase activity was calculated based on the definition of one unit of activity as the amount of enzyme that is used to produce 1 µmol reducing sugar per 20 min under the experimental conditions described above.

Characterization of alpha-amylase

Optimum pH

Several buffers, including Na-acetate buffer (pH 4.5–5.5), Na-phosphoric acid buffer (pH 6–6.5), and Tris-maleate buffer (pH 7) were used to maintain the pH of the reaction mixtures. The change in the enzymatic activity of the enzymes while incubating in these reaction mixtures was indicative of their optimum pH.

Optimum temperature and activation energy

The optimum temperature of α-amylase was determined by incubating an aliquot of the enzyme with 1% Papua sago starch at temperatures ranging from 60–95ºC in 0.1 M Na-acetate buffer for 20 min at pH 6.5. The activation energy (E_a) was calculated by using an Arrhenius plot (Siddiqui et al., 1996):

$k = A \cdot exp\left(\frac{-E_a}{RT}\right)$ or $\ln k = \ln A - E_a/\mathrm{R} \times T$

where k is the rate constant, R is the gas law constant, A is the Arrhenius constant, and E_a is the activation energy of the reaction. The R value is 8.31 J/mol K (gas constant), while T (absolute temperature) is in K. When ln k is plotted versus the reciprocal of T, a linear correlation was observed over the range of temperatures studied. The activation energy was calculated based on the slope of these lines.

Kinetics of starch hydrolysis

Initial reaction rates using sago starch as a substrate were determined at substrate concentrations ranging from 0.1–1.0% (w/v) in 0.1 M acetate buffer pH 6.5 at 85°C. The kinetic constants, i.e., V_{max}, K_m, k_{cat} and k_{cat}/K_m, were estimated using Lineweaver-Burk linear regression plots.

Thermodynamics of starch hydrolysis

The thermodynamic parameters for substrate hydrolysis were calculated by rearranging the Eyring's absolute rate equation derived from the transition state theory (Eyring and Stearn, 1939):

$$k_{cat} = (k_b T/h) e^{(-\Delta H^*)} \cdot e^{(\Delta S^*/R)}$$

where k_b is Boltzmann's constant (R/N) = 1.38×10^{-23} J/K, T the absolute temperature (K), h is Planck's constant (6.626×10^{-34} J s), N is Avogadro's number (6.02×10^{23} /mol), and R is the gas constant (8.314 J/mol K). Based on the activation energy (E_a) value and Arrhenius constant (A), different thermodynamic parameters such as enthalpy, entropy, Gibbs free energy, ΔH^*, ΔS^* and ΔG^*, respectively, were determined using the following equations:

$\Delta H^* = E_a - RT$
ΔG^* (free energy of activation) = $-RT \ln (k_{cat}h/k_b \cdot T)$
$\Delta S^* = (\Delta H^* - \Delta G^*) / T$

Determination of glucose concentration in the hydrolysate by using the glucose oxidase method

Hydrolysis was performed as described on section 2.4. The hydrolysate was cooled to room temperature, and then 0.2 mL of hydrolysate was mixed with 2 mL of a glucose oxidase-peroxidase (GOP) mixture (GOP and *o*-dianisidine dihydrochloride). Following incubation for 45 min at room temperature in the dark, absorbance at 425 nm was recorded and glucose concentration was determined based on extrapolation from a standard curve. The calibration curve comprised a standard glucose solution (Sigma) with concentrations between 25 and 75 µg/mL.

Determination of the dextrose equivalent (DE) value in hydrolysates

Dextrose equivalent (DE) was calculated based on the result of hydrolysis as measured by the Nelson-Somogyi method (section 2.5). The dextrose equivalent (DE) value is a measurement of the fraction of starch that is converted into glucose molecules. DE indicates the percentage of reducing sugars to starch weight. For example, a DE of 20 indicates that 20% of starch has been broken down into reducing sugars (Simms in Beynum and Roels, 1985).

HPLC Analysis of the malto-oligosaccharide composition in hydrolysates

According to the method reported by Batey (Batey, 1982), hydrolysates were read on a Brix hand-held refractometer to determine the appropriate value for measurement, and then 20 µL hydrolysate was injected into the HPLC, using ddH_2O as eluent with a flow rate of 0.6 mL/min. The HPLC system used was Sugar Analysis 1 (Waters Millipore), with an automatic sample injection module (Model WISP 712), pumps (Model 510), differential refractometer (Model 410), and MetaCarbAg column (MetaChem). The column temperature used was 85^{o}C. The software used for acquisition, storage, and data processing was ChromPerfect (ChromPerfect, USA). Identification of peaks and the concentration of each malto-oligosaccharides was performed by comparisons with standard solutions for HPLC, i.e., glucose (DP1–10; Sigma).

Results and Discussion

Effect of pH on α-amylase activity

A continual increase in enzyme activity was observed between pH 4.5 and the optimum pH 6.5 (Figure 1). Based on the mechanism of the hydrolysis reaction (Nielsen et al., 2001), residue Glu261 at the α-amylase active site is known to serve as a hydrogen (proton) donor. Thus, we estimated that, between pH 6.5 and pH 7.0, the environment around the active site becomes proton-deficient, which causes a decrease in the pKa of Glu261. This leads to a dramatic reduction of enzyme activity. Here, using the Nelson-Somogyi method, we found that the optimum pH was 6.5, which yielded a specific activity of 111.472 unit/mg (Figure 1 and Appendix 1). The optimum pH

was similar to the optimum pH observed in hydrolysis of corn starch by *B. licheniformis* α-amylase (BLA) (Vidilaseris et al., 2009). Vidilaseris et al. (2009) reported that marine bacterium α-amylase activity by *Bacillus* sp. ALSHL3 was optimal at pH 6.0. In contrast, Vengadaramana et al. (2011) reported that the activity of purified α-amylase from *Bacillus licheniformis* ATCC 6346 was optimal at pH 7.0.

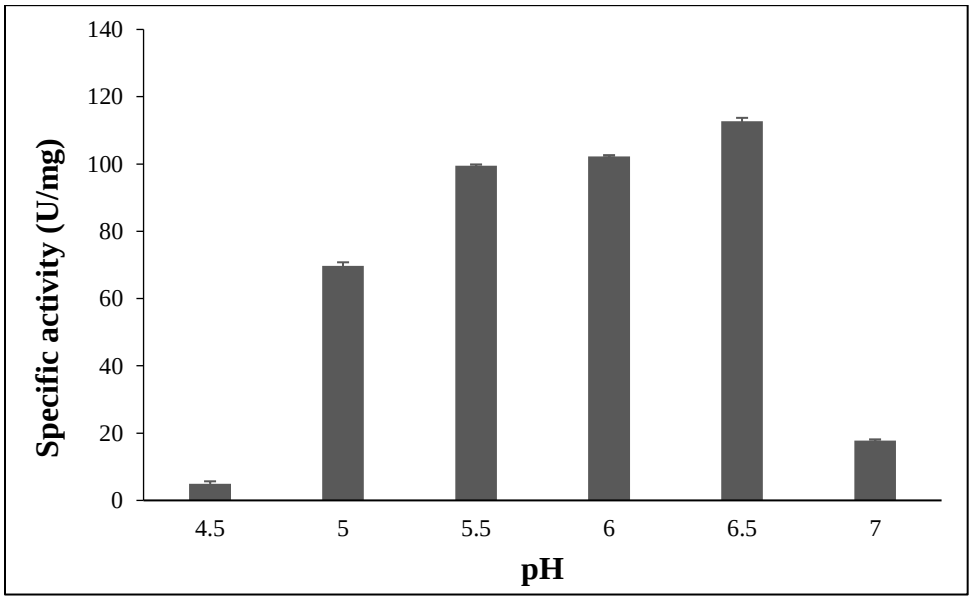

Fig 1. Effect of pH on enzyme activity

Optimum temperature, activation energy, and thermodynamics of sago starch hydrolysis

The enzyme (α-amylase) showed optimum activity at 85°C, which then declined at 90°C and 95°C (Figure 2). Here, we found that the activation energy (E_a) was 7.333 kJ/mol, as calculated from the Arrhenius plot. This result was very promising considering that the activation energy (E_a) was around 3 times lower than the activation energy of α-amylase from *B. licheniformis* mutant (25.14 kJ/mol) reported by Ul-Haq et al. (2010) when hydrolyzing of soluble starch. Meanwhile, activation energy of *B. licheniformis* α-amylase found by Duy and Fitter (Duy and Fitter, 2005) was around 363.7 kJ/mol at 70°C. It was higher than E_a value of *Aspergillus oryzae* α-amylase (317.9 kJ/mol). The free energy of the primary binding of this α-amylase at constant temperature (ΔG) was 50.843 kJ/mol, the enthalpy of activation (ΔH) was 4.855 kJ/mol, and the entropy of activation (ΔS) was -154.319 J/mol K (Appendix 3). The lower enthalpy value of enzyme could be due to a more efficient formation of the transition state (activated complex) between enzyme and substrate (Riaz et al., 2007). Ul-Haq et al. (2010) reported that the ΔG, ΔH, and ΔS for the binding of α-amylase isolated from *B. licheniformis* EMS-6 were 36.968 kJ/mol, 22.53 kJ/mol, and -110.95 J/mol K, respectively. Furthermore, the ΔH and ΔS values of *B. amyloliquefaciens* α-amylase reported by Tanaka and Hoshino (Tanaka and Hoshino, 2002) were 29.3 kJ/mol and -82.6 J/mol K, respectively.

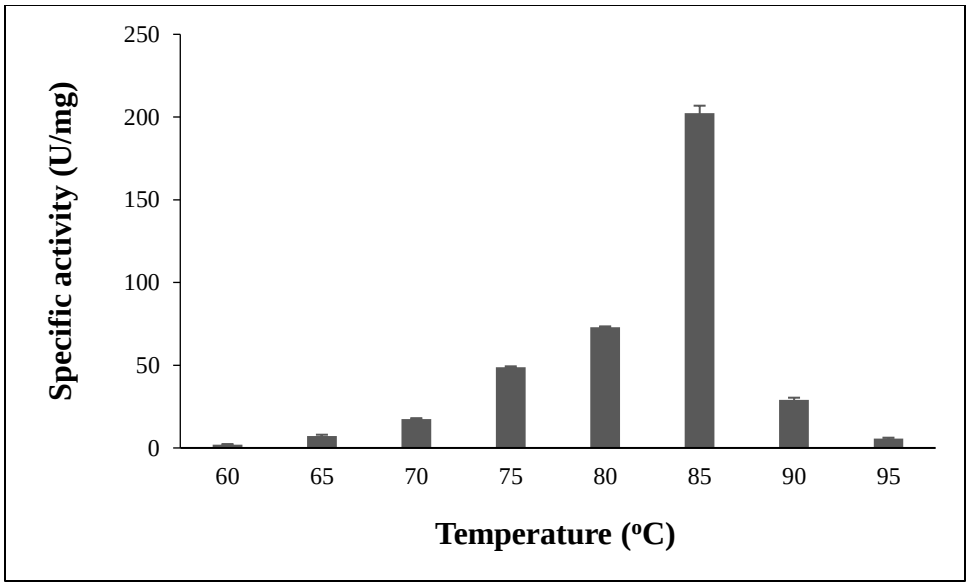

Fig 2. Effect of temperature on enzyme activity.

Kinetics of Papua sago starch hydrolysis by α-amylase

We determined the kinetic constants of reactions with α-amylase by incubating the same amount of enzyme with different concentrations of Papua sago starch as a substrate [0.1–1.0% (w/v)]. The enzyme activity profile exhibited Michaelis-Menten kinetics of catalysis. The values of K_m and V_{max} depend on the substrate used and the reaction conditions. Consequently, the K_m and V_{max} values of different enzymes are not directly comparable. Here, we found that the V_{max} and K_m of α-amylase, as determined from the Lineweaver-Burk plot, were 625 unit/mg and 2.19 % (w/v), respectively (Figure 3).

The high K_m value (2.19% (w/v)) revealed that Papua sago starch was not a highly specific substrate for the enzyme (α-amylase). Such low specificity is actually beneficial in industry, since the enzyme is thus able to hydrolyze more than one type of starch substrate. Therefore, the enzyme can also be utilized to hydrolyze other type of starch such as, corn starch, potato starch, rice starch etc., at the similar level of activity. Moreover, one and/or several types of starch can be combined or mixed together with sago starch as substrate. The specific activity of enzyme (unit/mg) used in this study, at least to some extent could display more informative and more specific results. It was also more difficult to find comparable references due to different reaction condition and/or different definition of one unit of enzyme activity. Nevertheless, one important factor of this study is, the substrate was raw sago starch which does not require many preparation and purification steps prior to the hydrolysis. Following reported reports also reveal some related information. The K_m and V_{max} values obtained from the hydrolysis of soluble starch by α-amylase from *B. amyloliquefaciens* were 4.11 mg/min and 3.076 mg at 50°C, respectively (Gangadharan et al., 2008). Goyal *et al*. (2005), reported that α-amylase from *Bacillus* sp. I-3 has a K_m value of 3.44 mg/mL and V_{max} value of 0.45 mg hydrolyzed starch/mL min at 50°C. Meanwhile, soluble starch hydrolysis conducted at 37°C and pH 8 using BLA from *E. coli* clones has been found to yield a K_m of 0.22% (w/v) (Somogyi, 1952). Additionally, analysis of Sulawesi sago starch hydrolysis

using α-amylase (Termamyl 120L) at 75°C and pH 6.5 yielded a K_m of 0.32% (w/v) (Ansharullah, 1997). A low K_m value is indicative of a high affinity of the enzyme for the substrate (Hamilton et al., 1998). Immobilized α-amylase from *B. licheniformis* followed Michaelis-Menten enzyme kinetics. The enzyme has a V_{max} of about 506 U/mg of bead protein, and a K_m of about 5 µM (Rasiah adn Rehm, 2009). This V_{max} is in line with the values obtained using free α-amylase in this recent study (625 unit/mg).

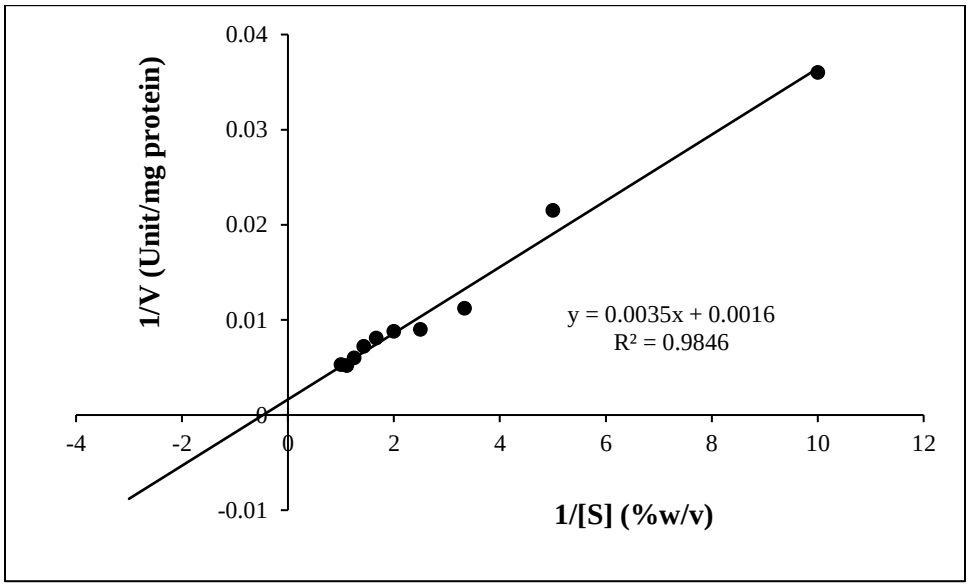

Fig 3. Lineweaver-Burk plot for the determination of kinetic constants of α-amylase, where the intercept on the y-axis corresponds to $1/V_{max}$ and the intercept on the x-axis to $1/K_m$. All data points are means of duplicates.

The turnover number (k_{cat}) is related to the maximum number of substrate molecules that are converted to product per active site per unit of time. The k_{cat}/K_m ratio is usually referred to as the 'specificity constant', which is an important value for comparing the relative rates of activity between enzymes utilizing different substrates (Eisenthal et al., 2007). Therefore, the k_{cat}/K_m ratio is an estimation of enzyme specificity between only two competing substrates, as reported by Fersht (1999). The key strategy usually used for maintaining sustainable activity is to produce an enzyme with increased catalytic efficiency (i.e., increased k_{cat}/K_m ratio). For extracellular enzymes that act at saturating substrate concentrations, such adaptation mainly consists of increasing k_{cat}. On the other hand, for intracellular enzymes, a decrease of K_m in order to provide higher substrate affinity could be useful. In the present study, k_{cat} was 7596.75/min and the specificity constant (k_{cat}/K_m) was 3468.34 mg/unit min. Nevertheless, for α-amylase from *Pseudoalteromonas haloplanktis*, the values of k_{cat}, K_m and k_{cat}/K_m, using 3.5 mM 4-nitrophenyl-α-D-maltoheptaoside-4,6-O-ethylidene as substrate, were 697/min, 234 µM, and 2.98 min/µM, respectively (D'Amico et al., 2002). The k_{cat} for α-amylase from *B. amyloliquefaciens* was 2.26×10^3 /min (Gangadharan et al., 2008).

Determination of malto-oligosaccharide composition in sago starch hydrolysate

HPLC analysis revealed that oligosaccharides with DP 1-10 were detected in the enzyme (α-amylase)-derived hydrolysate. Maltopentaose (DP-5) was the most dominant oligosaccharide found in the hydrolysate of Papua sago starch, followed by DP-3, and DP-2, while DP-8 was the least abundant oligosaccharide (Figure 4). Maltopentaose (DP-5) is a major product found in corn starch hydrolysate (Roussel et al.,1991). *B. licheniformis* α-amylase tends to hydrolyze substrates in a specific "endo" pattern, mainly at the fifth α-1,4-glycosidic bond from the reducing end (Labarge, 1988; Guzmán-Maldonado et al., 1995). This likely explains the abundance of DP-5 among the oligosaccharides.

The oligosaccharide composition of maltodextrin is a critical element in many food products, particularly since maltodextrin plays a major role in determining their sensory properties. These results indicate that the raw Papua sago starch used as substrate in this study has potential as an alternative cheap raw material in the maltodextrin industry mainly for its application as fat replacer in some food products.

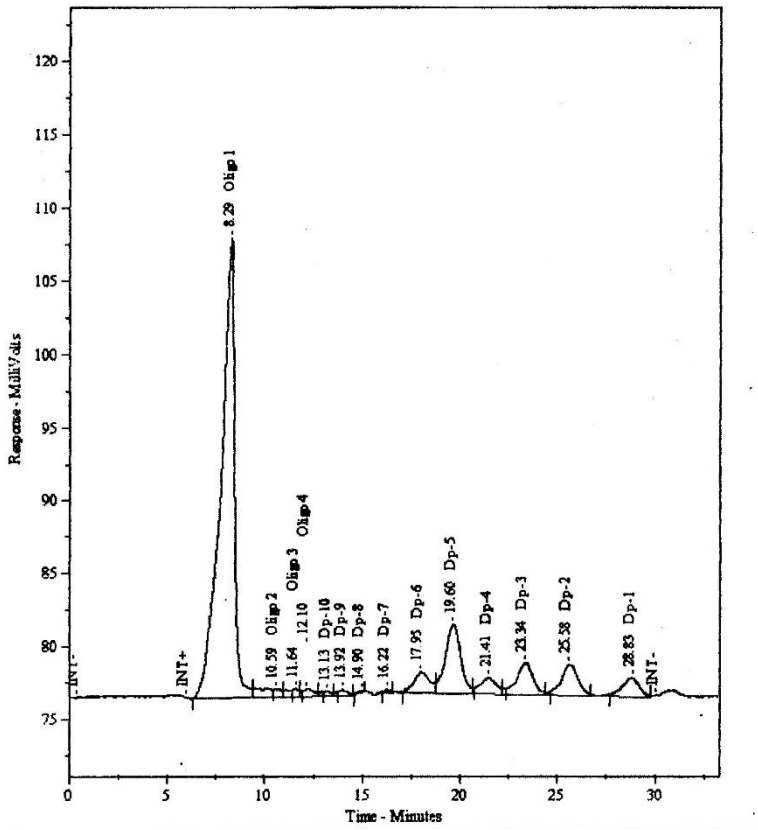

Fig 4. Chromatogram of oligosaccharides analyzed in the hydrolysate of sago starch

Dextrose equivalent value in hydrolysate

The DE value of Papua sago starch hydrolysate under the optimum conditions was 16.51% (Figure 5). This indicates that Papua sago starch hydrolysate has potential for use as an alternative raw material for maltodextrin production. This is feasible because maltodextrin which is widely used

in the industry has a DE of less than 20% (Marchal et al., 1999). We also found that the level of glucose produced from raw Papua sago starch hydrolysis in optimum conditions was 27.568 µg/mL.

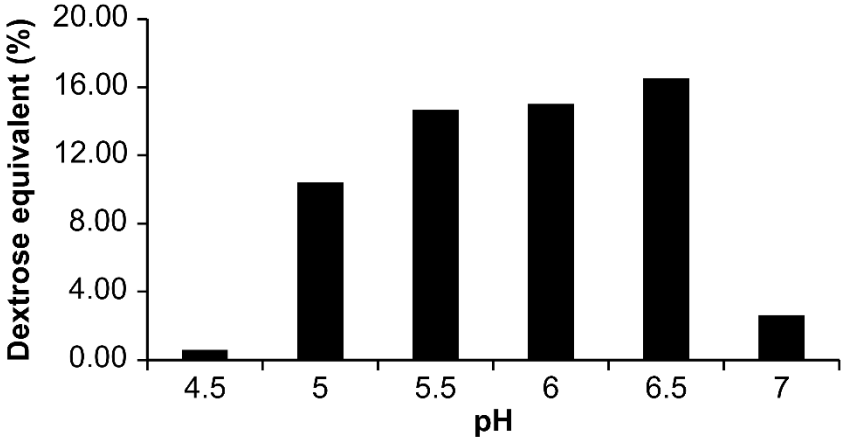

Fig 5. DE value of hydrolysate of Papua sago starch produced at different pH values at the optimum temperature.

Conclusions

The *Bacillus licheniformis* α-amylase used for the hydrolysis of Papua sago starch (*Metroxylon sagu* Rottbol) has an optimum temperature and pH of 85^{o}C and 6.5, respectively. The activation energy was 7.333 kJ/mol. The free energy of the system at constant temperature was 50.843 kJ/mol, enthalpy was 4.855 kJ/mol, and entropy was -154.319 J/mol K. The dextrose equivalent of hydrolysate was 16.51 %. The α-amylase has a K_m of 2.19 % (w/v) and a V_{max} of 625 units/mg. The glucose concentration produced under optimum conditions for hydrolysis was 27.568 µg/mL. Maltopentaose (DP-5) was a dominant species amongst the other malto-oligosaccharide in the hydrolysate. Papua sago starch could thus be used as an alternative cheap raw material in the maltodextrin industry.

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Amino Acid Profile of *Kappaphycus Alvarezii* And *Gracilaria Salicornia* Seaweeds from Gerupuk Waters, West Nusa Tenggara (NTB)

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Abstract

This research aimed to analyze the amino acid profile of *Kappaphycus alvarezii* dan *Gracilaria salicornia* seaweeds from Gerupuk Waters, West Nusa Tenggara (*NTB*). The amino acid analysis was performed using High-Performance Liquid Chromatography (HPLC). The results of the analysis showed that there were 15 amino acids contained in *Kappaphycus alvarezii* seaweed, consisting of 7 essential amino acids and 8 non-essential amino acids. Meanwhile, *Gracilaria salicornia* seaweed contains 13 amino acids, consisting of 7 essential amino acids and 6 non-essential amino acids. In *K. alvarezii,* the highest level amino acid was glutamate (13.73%) while in *G. salicornia,* the highest level amino acid was serine (13.02%). The results of the nutritional evaluation indicate that lysine is a limiting amino acid in *K. alvarezii* seaweed and methionine is a limiting amino acid in *G. salicornia* seaweed.

Keywords: Amino acid, Seaweed, K. alvarezii, G. salicornia, Gerupuk Waters

Introduction

Seaweeds are an important marine biological resource with high nutritional value, and thus it becomes one of the most promising plants in the future. With its unique biochemical structure and composition, seaweeds are widely used in various industries such as those engaged in the sectors of foods, energy, medicines, and cosmetics, as well as utilized for biotechnology (Norziah and Ching, 2000; Ommee and Payap, 2012; Komalavalli and Lalitha, 2015). Most seaweeds contain essential amino acids which are almost identical to the essential amino acids found in eggs (Norziah and Ching, 2000). Besides, most seaweeds contain quite a lot of aspartic acid, glutamic acid, and arginine in the composition of total amino acids (Rajasulochana *et al.*, 2010). The varied nutritional composition of seaweed is affected by the species, habitats, and environmental conditions (Zawawi *et al.*, 2014).

Kappaphycus alvarezii and *Gracilaria salicornia* are two species of seaweed fall under the class of *Rhodophyceae,* largely found in Gerupuk Waters, West Nusa Tenggara (*NTB*). *K. alvarezii* is a species of seaweed with a high economic value that has been widely cultivated as a source of carrageenan (Madhavarani and Ramanibai, 2014). Meanwhile, *G. salicornia* has the potential to

produce jelly, and its high chlorophyll content is potential as an antioxidant compound (Vijayavel and Martinez, 2010; Vimala and Poonghuzhali, 2015).

The results of Xiren and Aminah (2017) study suggest that *K. alvarezii* originating from Langkawi and Sabah Waters, Malaysia contains 19 amino acids, in which aspartic acid, glutamic acid, alanine, and leucine are the dominant amino acids. On another side, the information on the amino acid content of *Gracilaria* sp. genus suggests that the aspartic acid and glutamic acid of this species of seaweed are present in a dominant number (Norziah and Ching, 2000; Kumar and Kaladharan, 2007).

The amino acid composition of *K. alvarezii* and *G. salicornia* seaweeds in Gerupuk Waters, West Nusa Tenggara (*NTB*) has not been well known. Therefore, this research was intended to analyze the amino acid profile of *K. alvarezii* dan *G. salicornia* seaweeds from Gerupuk Waters, *NTB* so as to provide information on the nutritional value of both species.

Materials and Methods

Sampling Method

Seaweed samples used in this research were *K. alvarezii* derived from Gerupuk Waters, *NTB* at the ordinate of **8º55'3,46"S Lat. dan 116º20'3,49" E Lon.** and *G. salicornia* collected from the same waters at the ordinate of **8º55'1,4" S Lat. dan 116º19'57,22 E Lon.** The sampling of both seaweed samples was conducted in January. Both seaweed species were taken in fresh condition. Furthermore, both seaweed samples were inserted in polybag plastics, stored in small or fragmentary ice, and placed in a cool box. These seaweed samples were immediately taken to the laboratory. In the laboratory, the seaweed samples were washed from the dirt with clean water. After washed, they were wiped with tissue papers to remove the excess water that was still attached. The seaweed samples were then dried using an oven at 37º C until it gained a constant weight. The dried sample was mashed using a blender and sieved to obtain the sample powder to be then stored in a refrigerator (Lima-Filho *et al.*, 2002).

Analysis of Amino Acids

The amino acid analysis was performed using High-Performance Liquid Chromatography (HPLC). As much as 1 g of each sample was put into the reaction tube, then added with 4 ml of HCl 6 N and refluxed for 24 hours at 110º C. After that, the samples were cooled and neutralized with NaOH 6 N to pH 7, then filtered with 0,2 µm Whatman paper. As much as 10 µL of each sample was taken and added with 300 µL OPA. The samples were then left for 1 minute for perfect derivation. After that, as much as 20 µL of the sample was injected into the HPLC column and waited until the separation of all amino acids was complete. The standard amino acid solution used in HPLC consisted of 16 types of standard amino acids, namely aspartic acid, glutamic acid, asparagine, serine, histidine, glycine, threonine, arginine, alanine, tyrosine, methionine, valine, phenylalanine, isoleucine, leucine, and lysine.

The degree of each amino acid contained in both seaweed samples was determined by making standard amino acid curves. Meanwhile, the amino acid scores were determined using this following formula:

$$Amino\ Acid\ Score\ of\ the\ Sample\ = \frac{Essential\ Amino\ Acid\ Content\ of\ the\ Sample}{\text{FAO / WHO}\ Standard\ Amino\ Acid\ Pattern}$$

Data Analysis

The amino acid data found were analyzed descriptively and presented in the form of narration and table.

Results and Discussion

The results of the amino acid analysis on *K. alvarezii* and *G. salicornia* seaweeds in Gerupuk Waters, West Nusa Tenggara (*NTB*) can be seen in Table 1. Based on the results of the amino acid analysis using HPLC (Table 1), there were 15 amino acids contained in *K. alvarezii* seaweed, consisting of 7 essential amino acids and 8 non-essential amino acids. Meanwhile, in the content of *G. salicornia* seaweed, there were 13 amino acids identified, consisting of 7 essential amino acids and 6 non-essential amino acids. Both *K. alvarezii* and *G. salicornia* seaweed samples had the same essential amino acid profile, including Histidine, Leucine, Threonine, Valine, Methionine, Isoleucine, and Phenylalanine. However, both seaweed samples differed in the non-essential amino acid profile in which aspartate and lysine were present only in *K. alvarezii* (did not exist in *G. salicornia*). According to previous studies on the amino acid profile of *K. alvarezii* seaweed originating from Brazil (Gressler *et al.*, 2010) and Malaysia (Matanjun *et al.*, 2009), there are 16 amino acids obtained, consisting of 9 essential amino acids and 7 non-essential amino acids. Gressler *et al.* (2010) stated that the profile of amino acids can determine the nutritional quality of food, especially for the evaluation of new protein sources. Amino acid content is linearly dependent on seaweed protein content in which low protein content also causes low amino acid content. Therefore, the factors affecting amino acid content are also similar to those affecting protein content, covering species, environment, and harvest age (Hardjani *et al.*, 2017).

Table 1. Amino Acid Composition of *K. alvarezii* and *G. salicornia* Seaweeds in Gerupuk Waters, *NTB*

No.	Amino Acids	Percentage (% b/b)	
		K. alvarezii	***G. salicornia***
	Essential Amino Acids		
1	Histidine	1.01	7.92
2	Threonine	9.9	7.51
3	Methionine	1.86	1.11
4	Valine	9.94	7
5	Phenylalanine	6.05	2.94
6	Isoleucine	6.37	4.85
7	Leucine	8.43	6.6
	Total EAA	43.56	37.93
	% of total AA	47.58	46.49
	Non-Essential Amino Acids		
8	Aspartic Acid	12.26	-

9	Glutamic Acid	13.73	11.93
10	Serine	4.82	13.02
11	Glycine	7.71	5.85
12	Arginine	2.04	2.34
13	Alanine	7.33	9.11
14	Tyrosine	0.06	1.4
15	Lysine	0.05	ND
	Total Non-EAA	48	43.65
	% f total AA	52.42	53.51
	Total AA	91.56	81.58
	EAA/ Non-EAA	0.91	0.87
	EAA/Total AA	0.48	0.46

Note : AA, Amino acid
EAA, Essential amino acid.
Non-EAA, Non-essential amino acid.
ND, not determined.

The percentages of the total essential amino acids contained in *K. alvarezii* and *G. salicornia* seaweeds were respectively 47.58% and 46.49% of the total amino acids. This result is in line with Dawczynski *et al.* (2007) finding that the essential amino acid content of some types of seaweed was 30% of the total amino acids. Several previous studies have also reported that the total essential amino acids of *Caulerpa lentillifera* and *U.Reticulata* account for nearly 40% of the total amino acids (Ratana-arporn & Chirapart, 2006), those of *Kappaphycus alvarezii* dan *Hypnea musciformis* reach 45-49% (Dawczynski *et al.*, 2007; Kumar & Kaladharan, 2007), and those of *Gelidium pusillum* reach 52.08% (Siddique *et al.*, 2013).

The results of this research also showed that glutamic acid (13.73%) and aspartic acid (12.26%) contained in *K. alvarezii* seaweed were the highest level amino acids. On the other hand, the highest level amino acids of *G. salicornia* seaweed were glutamic acid (11.93%) and serine (13.02%). Several previous studies have also shown that aspartic acid and glutamic acid in red seaweed range from 15% - 33% (Matanjun *et al.*, 2009; Ortiz *et al.*, 2009; Gressler *et al.*, 2009). The content of aspartic acid and glutamic acid in red and green seaweeds is significantly higher than that of brown seaweed. The high content of aspartic acid and glutamic acid contributes to providing a distinctive flavor to seaweed, such as savory flavor (*umami* flavor) that can be used as food seasonings to stimulate the elderly's appetite or other diets. Meanwhile, serine can provide a sweet taste that causes a complex flavor to seaweed (Yaich *et al.*, 2011; Mouritsen, 2012). Furthermore, Holds and Kran (2011) explained that red algae contain many glutamic acids. The taste of *nori* (dried or edible seaweed often used in Japan cookery) is made up of the large number of different types of amino acids, including alanine, glutamic acid, and glycine.

The high percentage of glutamic acid, aspartic acid, and serine in *K. alvarezii* and *G. salicornia* seaweeds can be used as the consideration in the utilization of other potentials of both seaweeds, especially in the health sector. Khanifar *et al.* (2011) argued that glutamic acid is a precursor of glutamine and GABA. Aspartic acid helps to protect the liver and is involved in the metabolism of DNA and RNA. Moreover, it plays a role in the immune system by increasing the

production of immunoglobulins and the formation of antibodies. Meanwhile, serine is essential in maintaining blood sugar levels, plays a role in the central nervous system, including the development of the myelin sheath and is necessary for muscle growth and maintenance.

After the amino acid composition of *K. alvarezii* and *G. salicornia* (Table 1)was obtained, the next step was evaluating the nutritional value by comparing the essential amino acid content of the samples with the amino acid reference pattern recommended by FAO/ WHO (1991). This is particularly helpful in initial estimation of the nutrient content of seaweed protein (Kumar and Kaladharan, 2007; Siddique *et al.*,2013; Xiren and Aminah, 2017).

Amino acid score data presented in Table 2 below show that lysine is a limiting amino acid in *K. alvarezii* seaweed and methionine is a limiting amino acid in *G. salicornia* seaweed due to its lowest chemical value. Meanwhile, threonine is the amino acid with the highest chemical score in both *K. alvarezii* and *G. salicornia* seaweeds.

Table 2. Essential Amino Acids (mg/g protein) in *K. alvarezii* and *G. salicornia* Compared to the Amino Acid Reference Pattern Recommended by FAO (1991).

Essential Amino Acids	***K. alvarezii***	***G. salicornia***	**FAO/WHO (1991)**
Isoleucine	22.75	17.32	28
Leucine	12.77	10	66
Lysine	0.08	ND	58
Phenylalanine + Tyrosine	9.70	6.89	63
Methionine	7.44	4.44	25
Threonine	29.11	22.09	34
Valine	28.4	20.00	35

Note : ND, not determined

The results of Kumar and Kaladharan (2017) study suggest that leucine and lysine are limiting amino acids in *K. alvarezii* while threonine and tryptophane are the highest. On another side, lysine and methionine are limiting amino acids in *Gracilaria sp* while threonine and tryptophane are the highest. Furthermore, Xiren and Aminah (2017) study shows that lysine is a limiting amino acid in *K. alvarezii* seaweed species from both Langkawi and Sabah Waters in Malaysia.

In general, it can be seen that the content of *K. alvarezii* and *G. salicornia* seaweeds is rich in aromatic amino acids *i.e.* threonine and has a limited sulfur amino acid *i.e.* lysine. This suggests that *K. alvarezii* and *G. salicornia* seaweeds can be a source of complementary protein for human and animal needs.

Conclusion

1. *Kappaphycus alvarezii* seaweed contains 15 amino acids, consisting of 7 essential amino acids namely *Histidine (1.01%), Leucine (8.43%), Threonine (9.90%), Valine (9.94%), Methionine (1.86%), Isoleucine (6.37%), and Phenylalanine (6.05%)* as well as 8 non-essential amino acids namely Serine (4.82%), Aspartate (12.26%), Arginine (2.04%), Lysine (0.05%), Glutamic Acid (13.73%), Glycine (7.71%), Alanine (7.33%) and Tyrosine (0.06%). As for the content of *Gracilaria salicornia,* it was obtained 13 amino acids, consisting of 7 essential amino acids namely *Histidine (7.92%), Leucine (6.60%), Threonine (7.51%), Valine (7.0%), Methionine (1.11%), Isoleucine (4.85%), and Phenylalanine (2.94%)* as well as 6 non-essential amino acids namely *Serine (13.02%), Arginine (2.34%), Glutamic Acid (11.93%), Glycine (5.85%), Alanine (9.11%) and Tyrosine (1.40%).*
2. Both seaweeds differ in the non-essential amino acid profile in which aspartate and lysine are presented only in *K. alvarezii* seaweed (does not exist in *G. salicornia* seaweed).
3. Glutamate is an amino acid with the highest level (13.73%) in *K. alvarezii* seaweed while serine is an amino acid with the highest level (13.02%) in *G. salicornia* seaweed.
4. The results of the nutritional evaluation indicate that lysine is a limiting amino acid in *K. alvarezii* seaweed and methionine is a limiting amino acid in *G. salicornia* seaweed.

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Anther Development and Ultrastructure Pollen of *Spathodea campanulata* Beauv.

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Abstract

Anther development and ultrastructure pollen of *Spathodea campanulata* was studied as a part of completeness in embryology, especially family Bignoniaceae. Our studied showed the type of anther was tetrasporangiate. Anther wall consists of epidermis, middle layer (4 layers of cells), and tapetum (outer and inner tapetum). The type of tapetum was amoeboid. Ubisch bodies were observed on the tapetum cells. The process of microsporogenesis in *S. campanulata* was divided into 6 stages of anther development: undifferentiated anther stage, sporogen cells stage, microspore mother cell (MMC) stage, meiosis of microspore mother cell stage, tetrad stage and mature pollen. Microsporogenesis occur simultaneously, resulting tetrahedral tetrad. The type of pollen was tricolpate and have reticulate ornamentation.

Keywords: anther development, ultrastructure, pollen, S. campanulata

Introduction

Spathodea is a monotypic genus in the Bignoniaceae family, consisting of one species of *Spathodea campanulata* (Panga, 2014). S. *campanulata* is commonly known as the African tulip tree (Adeneye, 2014). It is important as a park and street tree, valued for its shade and spectacular flowers. It is used for soil improvement, reafforestation, erosion control, and as a live fence. It also has potential for greater use in rehabilitating disturbed lands on account of its pioneering ability and rapid growth. It is used in agriculture and forestry and as a medicinal plant (Bosch, 2002).

Anther development and ultrastructure pollen of *S. campanulata* is studied as a part of completeness in embryology. However, in Bignoniaceae these studies are limited (Mehra and Kulkarni 1985; Galati and Strittmatter 1999; Bittencourt and Mariath 2002; Konyar, 2014; Konyar, 2017). So far, no information on the embryology study of genus *Spathodea* have been published. The primary aim of this study is to determine whether the anther development and pollen type in *S. campanulata*.

The word 'anther' comes from the Greek *anthos*, which means a flower. It is the apical part of stamen (D'Arcy and Keating, 1996). Anthers structure varies from species to species and between different stage of development (Pacini, 1999). Microsporogenesis is an important process in anther, which includes several series of developmental stages from sporogenous cells to microspores (Yang and Kang, 2015; Ghimire et al., 2011; Bhojwani and Bhatnagar, 1999). In most

cases,the mature anther contain epidermal, endothecial, middle layer and connective cells (Pacini, 1999).

Pollen studies make important contributions to our knowlegde in many interdisciplinary arenas. Studies concerning pollen structure, size, and form are key issues in basic sciences, as, e.g. plant taxonomy and evolution, but are also are importance in applied fields as, e.g. plant breeding (Dafni et al., 2000). In Bignoniaceae family, eg *Campsis radicans* have spheroidal 3-colpate pollen grains with reticulate ornamentation and semi-tectate tectum (Konyar, 2014).

Materials and Methods

Plant Material

Fresh flower buds of different sizes and at different stages of development were collected from *S. campanulata* in Purwodadi Botanic Garden at November to December 2013.

Methods

For determine the process of microsporogenesis, material was prepared for LM using standard methods of paraffin embedding and serially sectioned using a rotary microtome (Soerodikoesoemo, 1987). Samples ranging from small buds to open flowers of different developmental stages were collected and fixed in FAA (formalin: glacial acetic acid: alcohol 70%) at 4°C for 24 h. The samples were dehydrated by alcohol series (70, 80, 90 and 95% and 100% (2x)), each for 30 minutes. The next step is dealcoholization, the samples were soaked in an alcohol/xylol mixture (3:1, 1:1, 1:3) and an absolute xylol (2x), each for 30 minutes. Then preserved in a mixture of xylol / paraffin (1:9) at 57^{o}C for 24 hours. The next step is infiltration, xylol/paraffin mixture were removed and were replaced with pure paraffin at 57^{o}C for 24 hours. The next step is cloaking using the new paraffin. After the paraffin hardened, it was blocked and affixed to the holder. Serial sections of thickness 10 µm were cut by rotary microtome. Staining was performed by 1% fast green for 30 seconds. All prepared slides were observed with the light microscope.

Scanning electron microscope (SEM) examination was conducted for observed the type of pollen. The samples were critical-point-dried using a critical point dryer, mounted onto pin stubs, coated with gold-palladium using a sputter coater, and examined using a Scanning Electron Microsscope (SEM) 'FEI tipe Inspects 25'. Transmission Electron Microscope (TEM) examination was conducted for observed the presence of the ubish bodies in the tapetum cells, as additional information of the microsporogenesis process in Spathodea. The procedure outlined of TEM analysis by Bozzola and Russell (1998) was followed. Sample of the anthers at the tetrad stage were fixed in 2.5% (w/v) glutaraldehyde in 0,1 M sodium cacodylate buffer (pH 7.4) for 24 h, 4^{o}C. The samples were rinsed with the same buffer three times for 15 minutes each. Then the samples were soaked in 2% osmium tetraoksida and 2,5% $K_3Fe(CN)_6$ in the same buffer for 2 h, 4^{o}C. The samples were rinsed again with the same buffer three times for 15 min each and dehydrated by passing them through a grade series of ethanol (30%, 50%, 70%, 95%) for 15 minutes each. This was followed by infiltration, embedding, cutting and colouring by triple lead. Then the samples

were examined under a Transmission Electron Microsscope (TEM) 'JEM 1010/ JEOL Electrone Microscope'.

Results and Discussion

Structure of the androecium

Androecium of *S. campanulata* has four epipetalous stamens, arranged in didynamous form (Fig. 1). Anthers were red, dorsifix, and tetrasporangiate. Anthers were shorter than the filaments. Mature anthers were 6-9 mm in length. When the anthers mature, the stomium opened longitudinally and pollen drop out.

Fig. 1. Androecium of *Spathodea campanulata* with didynamous stamens

Ontogeny of anther wall

Based on observation, the anther development in *S. campanulata* was divided into 6 stages, consisting of undifferentiated anther stage, sporogen cell stage, microspore mother cell stage, meiosis of microspore mother cell stage, tetrad stage, and mature pollen stage. The correlation between anther length and developmental stage was presented in Table 1 and Fig. 2.

Table 1. Correlation between the anther size of *S. campanulata* and the development stage.

Lenght of anther	Development stage	Note
0,5 mm	undifferentiated anther	Pic. 2.1
1-2 mm	sporogen cells	Pic. 2.2
2-2,5 mm	microspore mother cell (MMC)	Pic. 2.3
3-3,5 mm	meiosis of microspore mother cell stage	Pic. 2.4
4-5 mm	tetrad stage	Pic. 2.5
6-9 mm	mature pollen	Pic. 2.6

Fig.2. Anther of *S. campanulata* in different development stage

In cross sections, the undifferentiated anther of *S. campanulata* was observed as a homogeneous mass of cells surrounded by a single layer of epidermal (Fig.3.1). The epidermal performs its usual protective function. Some of the homogenous mass of cells were archesporial cells. They were signed by large size of cells dan more conspicuous nuclei (Fig.3.2).

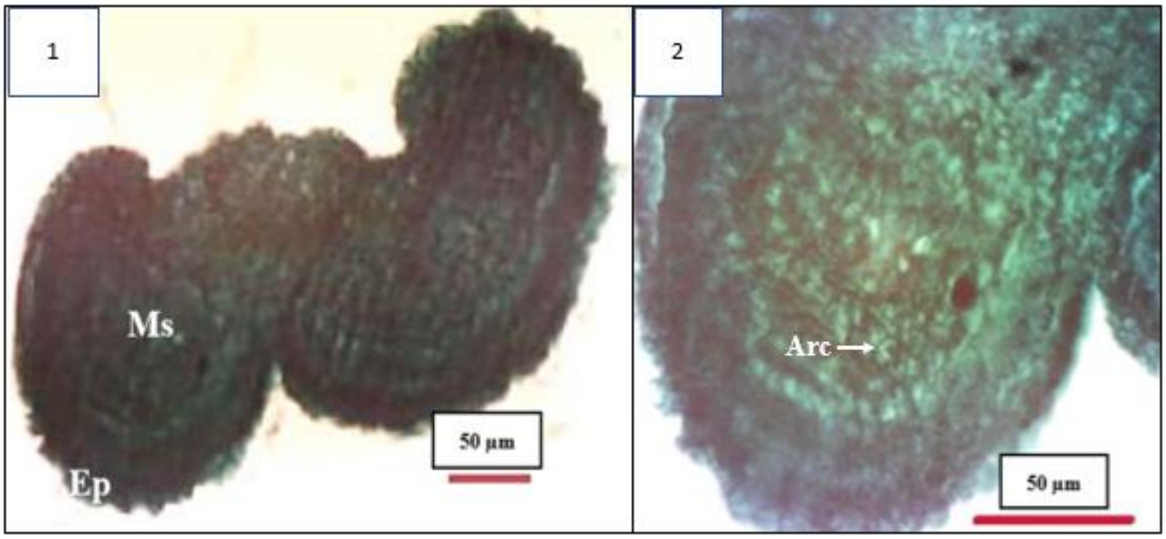

Fig.3. Cross section of anther wall formation in *S. campanulata*. (1) Single theca of the undiffentiated anther with single layer of epidermal and homogenous mass cell. (2) Homogenous mass cell with archesporial cells (*Ep* epidermal, *Ms* homogenous mass cells, *Arc* archesporial cell)

In the next stage (sporogen cells stage), the archesporial cells underwent periclinal divisions formed the primary parietal layer toward the outside and primary sporogenous layer toward the inside/connective site). Then the primary parietal layer also divided periclinally resulting the secondary parietal layer 1 (outer side/ towards the epidermis) and the secondary parietal cell 2 (inner side). Secondary parietal layer 1 returns divided periclinally several times resulting in 4 layers of middle layer cells, while the secondary parietal layer 2 will be directly developing into a tapetum cell on the outer side (outer tapetum) by vacuolization. In line with anther wall development, sporogenous cells also developed. The sporogenous cells consists of 2 rows of uninucleate cells curved like horseshoe shaped (Fig. 4.1, white arrow). The horseshoe shaped sporogenous cells common in other Bignoniaceae species (Konyar and Dane, 2013). The sporogenous cells differentiated and formed inner tapetum. At this stage, the cells of inner tapetum

seen bigger than the outer tapetum (Fig. 4.2). Following the development of the tapetum, connective cells that located at the end of sporogenous cells differentiated into tapetum cells and joined to the outer and inner tapetum. At this stage, the sporogenous cells had been sorrounded by the continuous layer of tapetum from 2 origin.

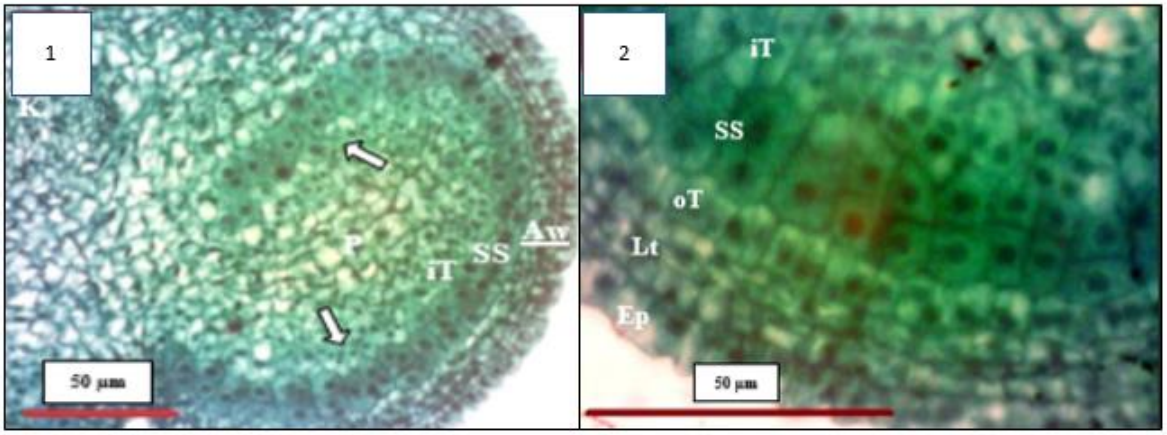

Fig.4. Cross section of anther wall formation in *S. campanulata* at sporogen cells stage. (1) The horseshoe shaped sporogenous cells. (2) Formation of anther wall (*Aw* anther wall, *P* parenchyma, *SS* sporogenous cells, *Ep* epidermal, *Lt* middle layers, *iT* inner tapetum, *oT* outer tapetum)

At the microspore mother cell (MMC) stage (anther range in length from 2 to 2.5 mm), the anther wall consist of three layers, the epidermal, middle layer, and the tapetum (Fig. 5.1). The sporogenous cells undergo development into microspore mother cell. The microspore mother cell looked clearer and bigger compared to the previous stage (Fig. 5.2, white arrow), so was the nucleus. It indicated that the cell ready to divided. At this stage, microspore mother cell and tapetum do vacuolization (Fig. 5.1, white arrows). Based on the observations, the size of inner tapetum cell larger than the outer tapetum cell.

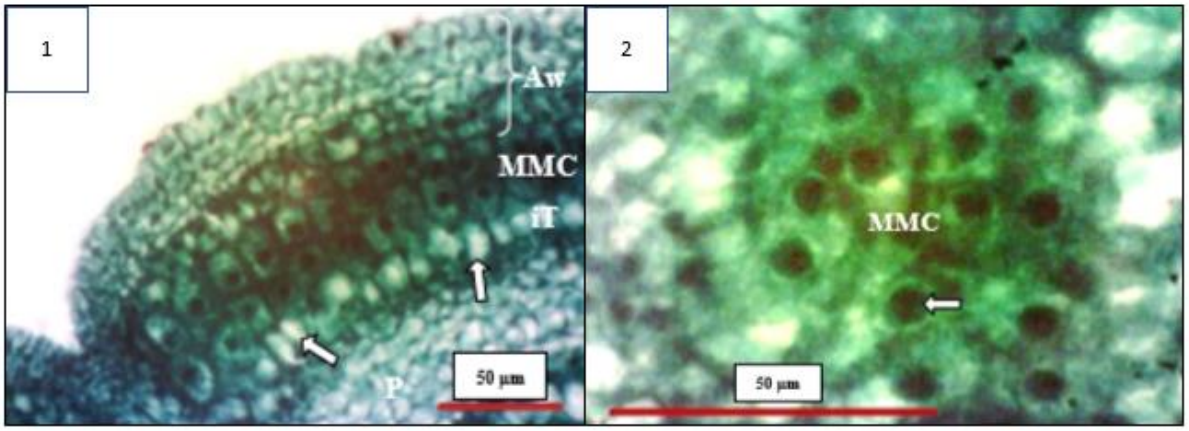

Fig.5. Cross section of anther wall formation in *S. campanulata* at microspore mother cell stage. (1) vacuolization of MMC and tapetum. (2) MMC ready to divided (*Aw* anther wall, *MMC* microspore mother cell , *iT* inner tapetum)

After outer and inner tapetum reached maximum growth, signed by vacuolization more bigger (Fig 6.1-2, white arrow), MMC enter meiosis, signed by microspore mother cell and nucleus looked

clearer and bigger compared to the previous stage (Fig 6.1, black arrow). At this stage, the anther range in length from 3 to 3.5 mm. In one locule, the development of each MMC could be different. By the time MMC complete their development, the tapetum cells begin to lose contact with each other. Large vacuole appear in the cytoplasm and the nuclei begin to show signs of degeneration. During this stage the middle layer become flattened and crushed (Fig 6.2 , black arrow).

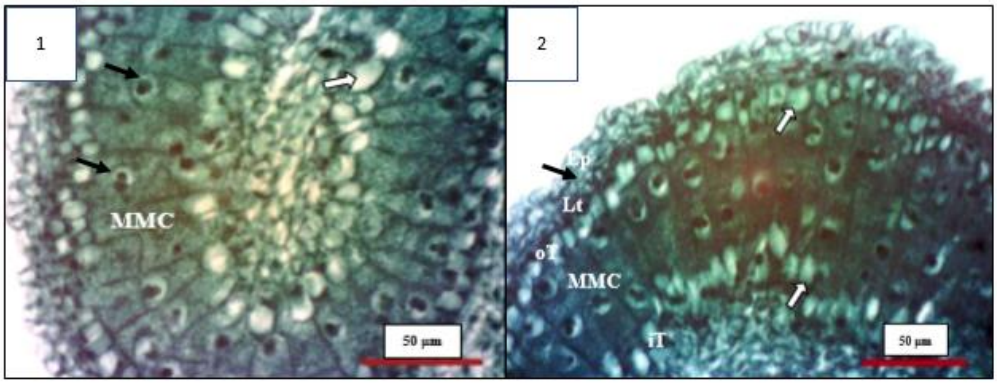

Fig.6. Cross section of anther wall formation in S. campanulata at meiosis of microspore mother cell stage. (1) vacuolization of MMC and tapetum. (2) MMC ready to divided (Aw anther wall, MMC microspore mother cell , iT inner tapetum, oT outer tapetum)

The divisions of MMC by cytokinesis resulted tetrad microspore with tetrahedral arrangement (Fig.7.5-6). At this stage (tetrad stage), the anther range in length from 4 to 5 mm. Tetrahendal type was also found in other species from Bignoniaceae family, e.g. *Campsis radicans* (Konyar and Dane, 2013). The tetrahedral type was a common type of tetrad in most plant species (Bhojwani and Bhatnagar, 1999; Maheshwari, 1950). In tetrad stage, the locule already formed, which the shape like a horseshoe (Fig. 7.1-2). Each side of anther consist of two locule. Along the anther locule enlarge, the middle layer become more flat compared to the previous stage (Fig. 3-4). All the spores in this stage were independent cells, no cellular communications were seen in loculus. Based on tapetum behaviour, it was catagorized as amoeboid tapetum. The tapetum cells lose their walls by the time the microspore tetrads have been formed (Fig. 7.4, black arrow). According to Furness and Rudall (2001) the transfer of nutrients from the amoeboid tapetum more efficiently than secretory tapetum.

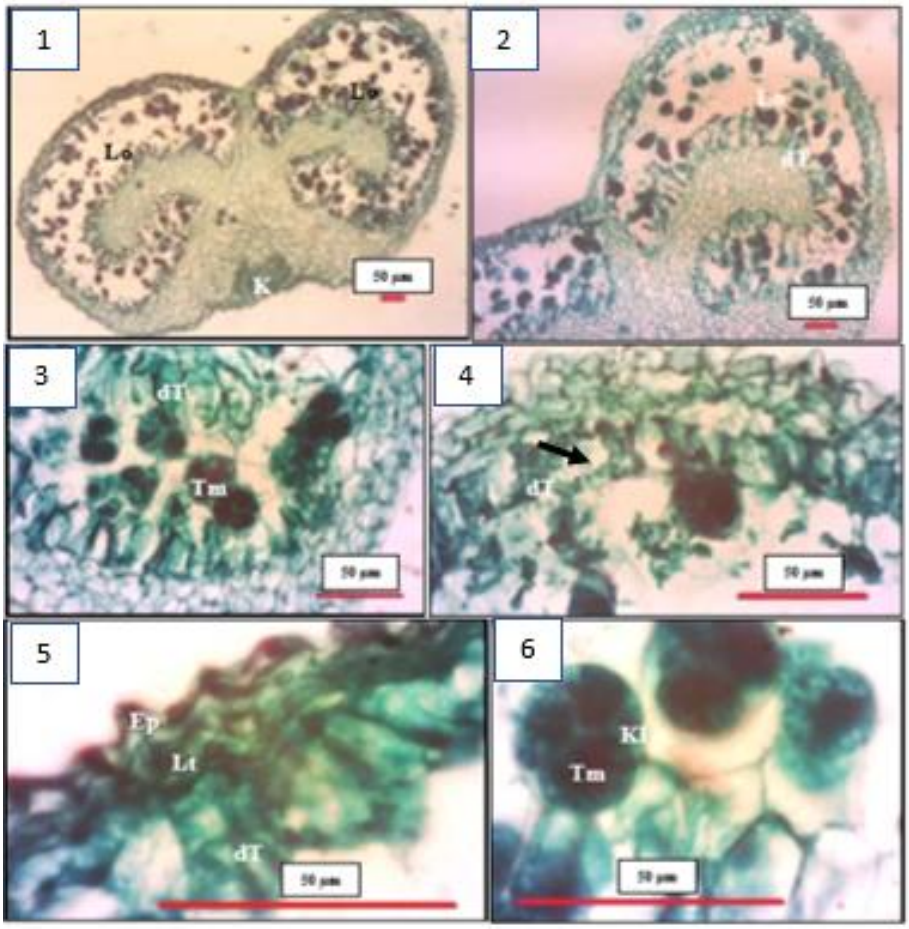

Fig.7. Cross section of anther wall formation in *S. campanulata* at tetrad stage. (1,2) locule formed, the shape like a horseshoe (3,4) degenerated tapetum (5) crushed middle layer (6) tetrad microspore (*Lo* locule, *K* connectivum, *dT* degenerated tapetum, *Tm* tetrad microspore, Kl callose, *Ep* epidermal, *Lt* middle layers)

Eventually, the microspores are set free in the another locule by the breakdown of the common callose wall which is supposed to be tapetum mediated. While still within the callose wall the microspores start synthesizing their individual walls. Callose seems to play an effective role in the laying down of the very first pattern of exine (Bhojwani and Bhatnagar, 1999).

Based on ultrastructure observation by Transmission Electron Microscope, ubisch bodies were observed on the tapetum cells in the early of tetrad stage (Fig. 8.1-2). When tapetum degenerated, Sporophytic proteins and ubish bodies will be transferred directly to fill the locule (Fig. 8.3-4)

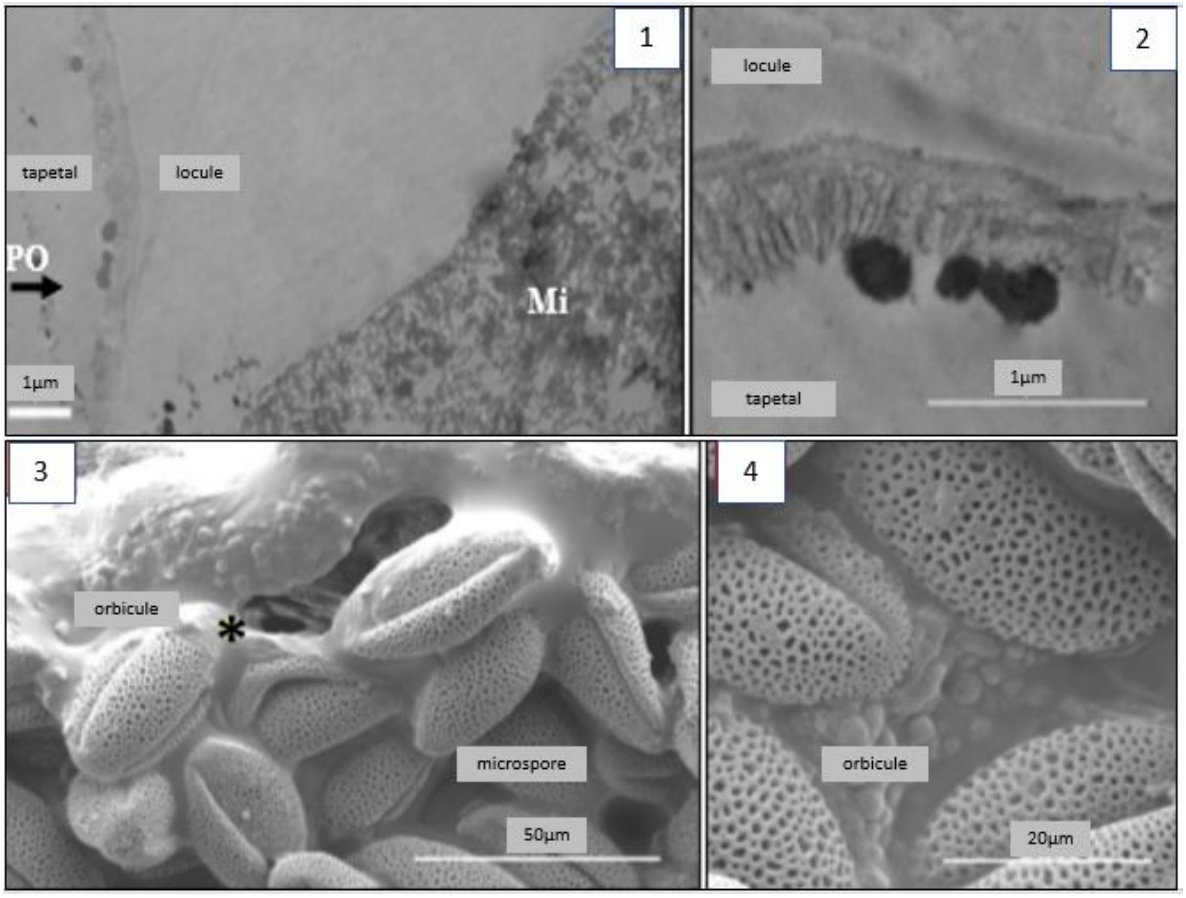

Fig. 8. The ubish bodies. (1,2) Observed using TEM. (3,4) Observed using SEM. *sporophytic proteins

At maturity, the two sporangia that were adjacent to each other joined due to the breakdown of the partition between them, to formed theca (Fig. 9.1). The anther locule become enlarge. In this stage, the anther range in length from 6 to 9 mm. The middle layer was crushed and flattened, it could be observed only ini some part of the anther (Fig. 9.2). When anther ready to dehiscence, some of epidermal cell (± 17 cells) near stomium formed exothecium (Fig. 9.3-4).

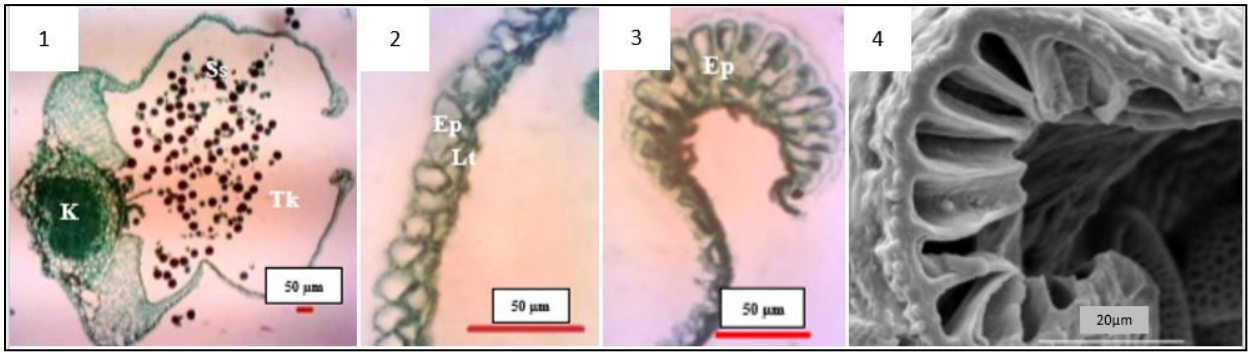

Fig.9. Cross section of anther wall formation in *S. campanulata* at mature pollen stage. (1) theca (2) layer (3) exothecium (4) exothecium observed using SEM (*Tk* Theca, *K* connectivum, *SS* pollen, *Ep* epidermal, *Lt* middle layers)

Under SEM, the pollen grains of *S. campanulata* were monads, isopolar and have reticulate ornamentation (Fig. 10, arrow). Isopolar pollen grains had identical proximal and distal poles, thus

the equatorial plane was a symetry plane. In the polar view, pollen grains seemed circular. Pollen grains length varied from a minimum of 60.5 µm to 67.4 µm and pollen grains width ranged from 34.9 µm to 44.2 µm. The shape was prolate (P/E 1.68).

An apertures was a region of the pollen wall that differs significantly from the rest of the wall in its morphology and/or anatomy, and was presumed to function usually as the site of germination and to play a role in harmomegathy. The character of apertures were combination of porus and colpus. Number, type and position of apertures were genetically determined and usually fixed within a species, however it may sometimes vary (Hesse *et al*., 2009). Based on the NPC-system (Number (N), Position (P) and Character (C) of apertures), apertures of pollen *K. pauciflora* were N_3 (tritreme), P_4 (zonotreme), and C_5 (colpate). It had three apertures with tricolpate type

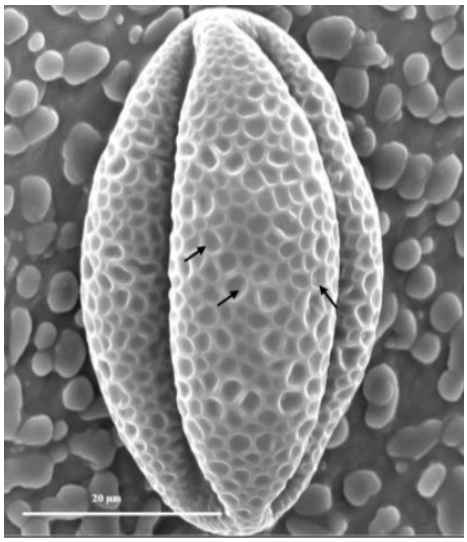

Fig. 10. Pollen of *S. campanulata* under SEM

Conclusion

Anther wall of *S. campanulata* consists of epidermis, middle layer (4 layers of cells), and tapetum (outer and inner tapetum). The type of tapetum was amoeboid. Ubisch bodies were observed on the tapetum cells. The process of microsporogenesis in *S. campanulata* was divided into 6 stages of anther development: undifferentiated anther stage, sporogen cells stage, microspore mother cell (MMC) stage, meiosis of microspore mother cell stage, tetrad stage and mature pollen. Microsporogenesis occur simultaneously, resulting tetrahedral tetrad. The type of pollen was tricolpate and have reticulate ornamentation.

Acknowledgments

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Box-Behnken Design of RSM Application to Optimize A Direct Sonication-Assisted Transesterification Process in Biodiesel Production from Marine *Chlorella* sp. Biomass

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Abstract

This study aimed to optimize biodiesel productions from marine *Chlorella* sp. biomass by direct sonication-assisted transesterification procedures. The method was optimized through Response Surface Methodology (RSM) with variations in important factors including reaction temperature (50-70^{o}C), chloroform:methanol volumetric ratio (0.35-2.35) and reaction time (60-120 min). Box-Behnken Design (BBD) was applied for the experimental design. The maximum biodiesel yield (38.6%) was achieved at reaction temperature of 70^{o}C, chloroform:methanol volumetric ratio of 1.35 and reaction time of 120 min. This method exhibits great potential in production of microalgal biodiesel. The response functions in the term of actual factor to predict biodiesel yield (Y_i) given in following equation: $Y_i = 201.72 – 4.79A – 15.85B – 0.85C + 0.09AB + 0.004AC + 0.09BC + 0.04A^2 – 0.09B^2 + 0.003C^2$, where independent variables: A is reaction temperature, B is chloroform:methanol volumetric ratio, C reaction time. The design of the experiment including dependent variables response Y_i is biodiesel yield, while A, B, C, D are referred as the main effort linear terms. AB, AC, AD, BC, BD, CD are the interaction terms. A^2, B^2, C^2, D^2 are the quadratic terms involved in the process.

Keywords: Box-Behnken Design (BBD), marine Chlorella sp., microalgal biodiesel, RSM, sonication-assisted transesterification

Introduction

There are several reasons make biodiesel as an important energy resource. First, as a renewable energy resource, biodiesel could be sustainably produced (Sheehan, *et al*., 1998; Mandik *et al.* 2015). Second, biodiesel has several properties which are environmentally friendly (Antolin *et al*., 2002; Vicente *et al*., 2004; Maithy *et al*., 2014; Gnansounou and Raman., 2016). Third,

economically, biodiesel has big potential because fossil fuel prices intend to increase predictably in the future (Cadenas and Cabezndo, 1998). Furthermore, biodiesel has several characteristics which are compatible with the petroleum diesel (fossil fuel). For instance, the higher heating value (HHV) of biodiesel (39–41 MJ/kg) is comparable with that of petrodiesel (43 MJ/kg). Other characteristics such as cetane number, kinematic viscosity, and flash point are also similar (Fuls *et al*., 1984; Knothe., 2010).

Microalgae are photosynthetic microorganism which can utilize sunlight and carbon dioxide to produce biomass, and oxygen. Microalgal cells develop and grow in water suspension, so in utilizing water, carbon dioxide, and other nutritions, microalgae have higher level of efficiency than plants (Deng *et al*., 2009; Widjaja, 2009; Caffarri *et al*., 2014). Microalgae are also known as microorganism with capability as biofuel factory, such as biodiesel (produced via transesterification reaction), bioethanol (produced via fermentation), hydrogen, and biogas (Basmal, 2008; Mandik *et al.* 2015).

Several procedures can be used for extracting lipids from microalgae, such as mechanical pressing, milling, homogenization, solvent extraction, ultrasonic-assisted extraction, supercritical fluid extraction, enzymatic extractions, and osmotic shock. Every method has its individual advantages and disadvantages (Mercer and Armenta, 2011). The use of a combination method with sonication method in lipid extraction potentially shows faster extractions and higher yields.

In this research, lipid in dry microalgal biomass was simultaneously extracted and transesterified by modified sonication-assisted method of Cheng *et al*. (2013)., and the process was optimized by using Box-Behnken Design of Response Surface Methodology (RSM).

Materials and methods

Microalgae strain and growth medium

Marine *Chlorella* sp. was obtained from the National Institute of Coastal Aquaculture, Thailand. The mediums used in this study were BG-11 medium (Cheirsilp and Torpee, 2012). One liter of BG-11 medium contains $NaNO_3$ 1.5 g, $K_2HPO_4 \cdot 3H_2O$ 0.04 g, $KH_2PO_4 \cdot 3H_2O$ 0.2 g, EDTA 0.0005 g, Fe ammonium citrate 0.005 g, citric acid 0.005 g, Na_2CO_3 0.02 g and 1 mL of trace metal solution, pH 7.3. One liter of trace metal solution contains H_3BO_3 2.85 g, $MnCl_2 \cdot 4H_2O$ 1.8 g, $ZnSO_4 \cdot 7H_2O$ 0.02 g, $CuSO_4 \cdot 5H_2O$ 0.08 g, $CoCl_2 \cdot 6H_2O$ 0.08 g and $Na_2MoO_4 \cdot 2H_2O$ 0.05 g.

Cultivation of microalgae

Microalgae strain were pre-cultured in 400 mL of BG-11medium in a 500 mL bottle. The pre-cultures were incubated at 30 ^oC and air-aerated at a flow rate of 0.01 mL/min under a 3,000 lux light intensity with a 16:8 h light and dark cycle for 7 days. Afterward, this culture was used as a seed culture. The batch cultivation of the microalgae was performed by inoculating 10% (v/v) seed culture into each 3 L of BG-11medium in a 3.78 L (1 US gallon) glass bottle. Cultures were incubated at 30 ^oC and air-aerated at a flow rate of 0.01 mL/min. The cultures then were illuminated with a 3,000 lux light intensity with a 16:8 h light and dark cycle for 5 days (Cheirsilp and Torpee, 2012).

Subsequently, microalgal cells were harvested by centrifugation at 4500 rpm for 15 min and dried at 60ºC to constant weight in vials, lipid in dry microalgal biomass was simultaneously extracted and transesterified by modified sonication-assisted method of Cheng *et al*. (2013). The produced microalgal biodiesel was used in GC analysis to evaluate its fatty acid composition and percent of FAME. Meanwhile, lipid extraction from 0.0625 g of dried microalgal biomass was also conducted based on modified sonication-assisted method of Cheng *et al*. (2013) for simultaneous extraction and transesterification, without sulfuric acid addition.

Optimization through Response Surface Methodology (RSM)

Based on the results from screening of transesterification methods, the selected method (method IV) was then used in further study. The effects of reaction temperature, volumetric ratio of chloroform/methanol, and reaction time, in direct sonication-assisted transesterification reaction of dry marine *Chlorella* sp. biomass were evaluated through Response Surface Methodology (RSM) in order to optimize biodiesel yield. Design Expert 8.0.6 (Stat-Ease, Inc., Minneapolis, MN, USA) was used for regression analysis of experimental data and to plot response surface with the experimental results of biodiesel yield from the all of design experimental setup. This experiment was based on a Box Benhken Design (BBD) with a quadratic model employed to study the combined effect of three independent variables, i.e. reaction temperature, volumetric ratio of chloroform/methanol, and reaction time in direct sonication-assisted transesterification reaction (method IV) of dry marine *Chlorella* sp. biomass and each variable varied at three levels (-1, 0, +1). The biodiesel yield was calculated and used as a dependent variable.

Table 1. Codes and actual levels of the independent variable for design of the experiment used in BBD method.

Run	Reaction Temperature (˚C)	chloroform:methanol volumetric ratio	Reaction Time (min)
1	(60) 0	(1.35) 0	(90) 0
2	(70) 1	(2.35) 1	(90) 0
3	(50) -1	(0.35) -1	(90) 0
4	(50) -1	(1.35) 0	(60) -1
5	(60) 0	(1.35) 0	(90) 0
6	(60) 0	(0.35) -1	(60) -1
7	(60) 0	(1.35) 0	(90) 0
8	(60) 0	(2.35) 1	(120) 1
9	(70) 1	(1.35) 0	(60) -1
10	(70) 1	(0.35) -1	(90) 0
11	(70) 1	(1.35) 0	(120) 1
12	(60) 0	(1.35) 0	(90) 0
13	(50) -1	(2.35) 1	(90) 0
14	(60) 0	(2.35) 1	(60) -1
15	(60) 0	(0.35) -1	(120) 1
16	(50) -1	(1.35) 0	(120) 1
17	(60) 0	(1.35) 0	(90) 0

Note: Values in parentheses are uncoded independent variables

Main sources to determine these criteria: Cheng *et al*., 2013, Neto *et al.*,2013, and this recent screening study.

The second order polynomial quadratic equation was fitted to evaluate the main effect and interaction of each independent variable to the response as given by equation 1.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i<j}^{n} \beta_{ij} X_i X_j + \sum_{j=1}^{n} \beta_{jj} X_j^2 \quad (1)$$

Where *Y* represent the response of experimental (biodiesel yield), i and j are linear and quadratic coefficients respectively, β is the regression coefficient, n is the number of variables studied in the experiments, and X_i is factors (independent variables), they are represent reaction temperature (^oC), volumetric ratio of chloroform/methanol, and reaction time (min) in direct sonication-assisted transesterification, respectively.

Results and discussions

Statistical analysis on biodiesel yield

The experimental design with the observed responses for biodiesel yield are presented in Table 2. Based on the experimental design, biodiesel yields were in range from 18.2 to 38.6 %. It was found that Run 11 (reaction temperature of 70 ^oC, chloroform:methanol volumetric ratio of 1.35, and reaction time of 120 min) gave the maximum biodiesel yield of 38.6 % followed by Run 10 (reaction temperature of 70 ^oC, chloroform:methanol volumetric ratio of 0.35, and reaction time of 90 min) gave the maximum biodiesel yield of 35.2 %. This result means that the comparable biodiesel yield could be obtained using a shorter reaction time (90 min at Run 10) using much more amount of methanol than chloroform.

Furthermore, the experimental data were analyzed using Design Expert software (8.0.6 version) for analysis of variance (ANOVA), regression coefficient, and regression equation. Sequential *P-value* was used to select the highest order polynomial where the additional terms are significant, and the model is not aliased. The suitable model that have insignificant lack-of-fit and maximum R-squared and maximum adjusted R-squared was selected (Table 3). The response functions in the term actual factor to predict biodiesel yield (Y_i) given in equation 2.

$$Y_i = 201.72 - 4.79A - 15.85B - 0.85C + 0.09AB + 0.004AC + 0.09BC + 0.04A^2 - 0.09B^2 + 0.003C^2 \quad (2)$$

Where independent variables: A is reaction temperature, B is chloroform:methanol volumetric ratio, C reaction time. The design of the present experiment including dependent variables response Y_i, was biodiesel yield A, B, C, D are referred as the main effort linear terms. AB, AC, AD, BC, BD, CD are the interaction terms. A^2, B^2, C^2, D^2 are the quadratic terms involved in the process.

Table 2. BBD with the experimental responses

Run	Factor 1 Reaction Temperature (°C)	Factor 1 chloroform:methanol volumetric ratio	Factor 1 Reaction Time (min)	Response Biodiesel Yield (%)
1	(60) 0	(1.35) 0	(90) 0	24.4
2	(70) 1	(2.35) 1	(90) 0	29.6
3	(50) -1	(0.35) -1	(90) 0	27.4
4	(50) -1	(1.35) 0	(60) -1	25.1
5	(60) 0	(1.35) 0	(90) 0	22.9
6	(60) 0	(0.35) -1	(60) -1	29.1
7	(60) 0	(1.35) 0	(90) 0	25.4
8	(60) 0	(2.35) 1	(120) 1	29.1
9	(70) 1	(1.35) 0	(60) -1	29.9
10	(70) 1	(0.35) -1	(90) 0	35.2
11	(70) 1	(1.35) 0	(120) 1	38.6
12	(60) 0	(1.35) 0	(90) 0	22.9
13	(50) -1	(2.35) 1	(90) 0	18.2
14	(60) 0	(2.35) 1	(60) -1	19.0
15	(60) 0	(0.35) -1	(120) 1	29.0
16	(50) -1	(1.35) 0	(120) 1	28.6
17	(60) 0	(1.35) 0	(90) 0	23.2

Note: Values in parentheses are uncoded independent variables

Table 3. Fit summary analysis of variance for independent variables on response variables

	p-value	R-Squared	Adjusted R-Squared	selection
Linear	0.0028	0.386658	0.567571	
2FI	0.4178	0.130701	0.571187	
Quadratic	0.0006	0.76298	0.941744	Suggested
Cubic	0.3346		0.952687	Aliased

Significant level was defined as $p<0.05$

The regression coefficients for biodiesel yields are described in Table 4. For biodiesel yield, the statistical significance of the model equation was analyzed by the F-test for analysis of variance (ANOVA), to the fitted model. The *P-value*s < 0.05 implies that model was statistically valid. The regression of linear term *B* (chloroform : methanol volumetric ratio) was the most significant factor of biodiesel yield, the *P-value* is 0.0020, followed by linear term *A* (reaction temperature) with *P-value* of 0.0102. Meanwhile, linear term *C* (reaction time) was not significant.

Table 4. Regression coefficients of response variables

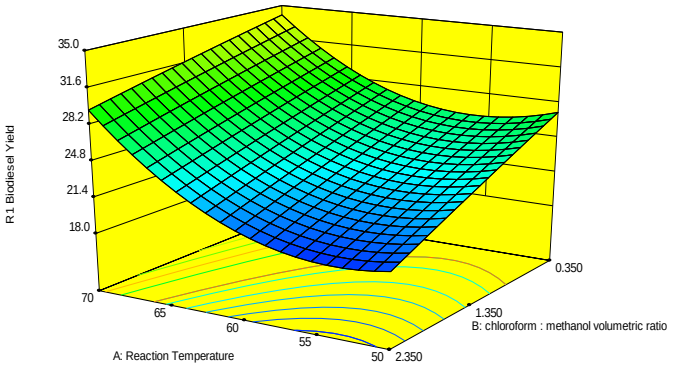
Source	Biodiesel yield	
	Coefficients	p-value
Intercept	201.72	0.0004
A-Reaction Temperature	-4.79	0.0102
B-chloroform : methanol volumetric ratio	-15.85	0.0020
C-Reaction Time	-0.85	0.2040
AB	0.09	0.0810
AC	0.004	0.0046
BC	0.09	0.0004
A^2	0.04	0.8881
B^2	-0.09	0.0022
C^2	0.003	0.0004

* means significant at 5% level

Response surface plots for biodiesel yield

The three-dimensional response surface and two-dimensional contour plots from the calculated responses in which one of variable was kept constant at its center point and the other two variables were varied within their experimental range, are shown in **Fig** 1. These graphs were plotted in order to investigate the interactions between independent variables and determine the optimal level of each variable for a desired response.

The interaction between chloroform : methanol volumetric ratio (B) vs. reaction time (C) with *P-value* of 0.0004, and the interaction between reaction temperature (A) vs. reaction time (C) with *P-value* of 0.0046 significantly influenced the biodiesel yield (*P-value* < 0.05). On the other hand, interaction of reaction temperature (A) vs. chloroform : methanol volumetric ratio (B) was not significant (*P*-value > 0.05). For quadratic terms B^2 with *P*-value of 0.0022 and C^2 with *P*-value of 0.0004 were significant.



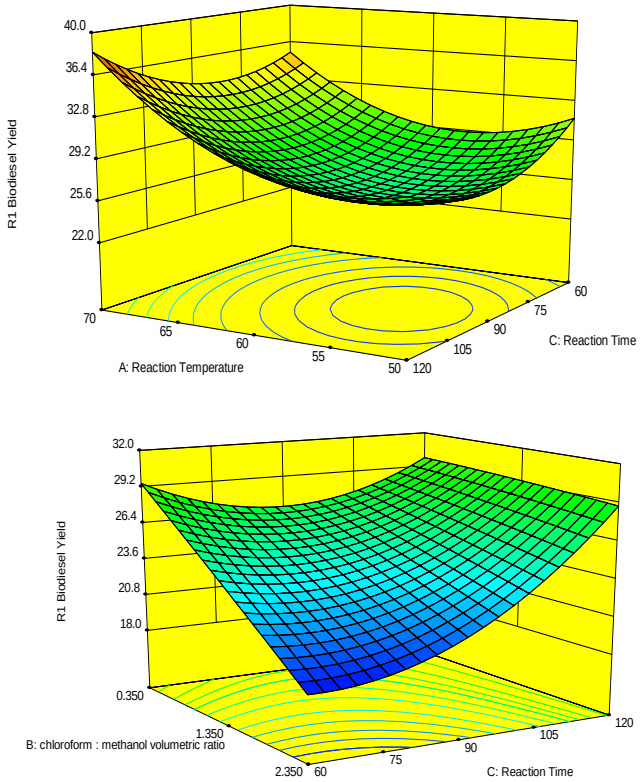


Fig 3. Contour and 3D of response surface plots of biodiesel yield. **Top**: Effect of reaction temperature and chloroform:methanol volumetric ratio. **Middle**: Effect of reaction temperature and reaction time. **Bottom:** Effect of chloroform:methanol volumetric ratio and reaction time

Conclusions

Marine *Chlorella* sp. cells which contain suitable lipids can be directly transesterified to produce biodiesel using sonication assisted-direct transesterification method. Optimization of this method gave maximum biodiesel yield (38.6%) which achieved at reaction temperature of 70^{o}C, chloroform:methanol volumetric ratio of 1.35 and reaction time of 120 min. This method exhibits great potential in the production of microalgal biodiesel.

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Chlorophylls, Lipid and Growth Characterization of Jayapura Freshwater Microalgae (*Scenedesmus sp.*) which Cultivated in A Cheap Fertilizer Medium

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Abstract

Microalgae were known as photosynthetic microorganism which has great potential to be developed as biodiesel feedstock. This research was conducted to isolate, to cultivate and to characterize potential species of freshwater microalgae from Jayapura, as Jayapura located close to equator therefore mostly exposes to sun light along year. The chlorophylls content, lipid content and growth rate of selected microalgae were characterized after cultivating in urea and NPK fertilizer liquid growth medium which is a low-cost medium. Several species of microalgae were isolated and identified as *Scenedesmus sp., Chlorella sp., Eudorina elegans* and *Nannochloropsis sp*. and *Zoochorella* sp. Furthermore, *Scenedesmus sp.*, which grew well were cultivated as the selected microalgae. Chlorophylls content of *Scenedesmus sp.*, harvested at the peak of growth rate were 0.4992 mg/L of Chlorophylls a and 2.1889 mg/L of Chlorophylls b, respectively. Meanwhile, the lipid content was 30% (w/w). Biodiesel was produced by using direct sonication-assisted transesterification method. The fatty acids compositions of produced microalgal biodiesel were as follows, palmitic acid (24.21%), linolenic acid (17.09%), linoleic acid (16.52%), stearic acid (12.82%), and elaidic acid (5.13%), respectively. Jayapura isolate of *Scenedesmus sp.*, exhibits great ability to grow in a cheap fertilizer containing medium. The lipids could be utilized as biodiesel feedstock while the chlorophylls are applicable as healthy food supplement and antioxidant products.

Keywords: biodiesel, cheap medium, chlorophylls, direct sonication-assisted transesterification, and Scenedesmus sp.

Introduction

Biodiesel has been known as one of the important energy resources produced from vegetables oil. Biodiesel could also be produced from microalgal lipid through transesterification reaction (Leung et al., 2010). Cetane number, flash point, higher heating value (HHV), and kinematic viscosity are several characteristics of microalgal biodiesel which are similar to petroleum diesel's (fossil fuel) (Knothe, 2010). This bioenergy is also sustainable, environmentally friendly, and economically potentials (Maithy et al., 2014; Slade and Bauen., 2013; and Gnansounou and Raman., 2016).

Microalgae can provide energy by conducting photosynthesis. They also grow so quickly. Some species can adapt and grow very well in various environments. It is obviously an advantage for further development of microalgae as biofuel's raw materials because they can be grown in various areas and the utilization of microalgae for biofuels does not compete with human consumptions (Vanthoor-Koopmans et al., 2013 and Slade and Bauen., 2013). During microalgae growth, lipids were synthesized and accumulated in microalgal biomass. Lipid content of microalgae varies from species to species in the range of 5 to 77 % of dry biomass (Liang et al., 2013 and Abomohra et al., 2016).

Chlorophylls is a green pigment which abundantly found in microalgae cells. This pigment mainly involves in photosynthesis process. There are two main types of chlorophylls which are important for capturing photon of light. Chlorophylls a as the primary photosynthetic pigment could capture light with wave length of 430 nm (blue) to 663 nm (red). Chlorophylls a were mostly produced in photosystem I. Meanwhile, chlorophylls b could capture light with wave length of 453 nm to 642 nm. Photosystems II contains more chlorophylls b than chlorophylls a and in less light conditions chlorophylls b were produced more to increase photosynthesis (Caffarri et al., 2014).

Papua province of Indonesia has been known for its abundance of marine and freshwater microorganism due to the mega biodiversity. However, the scientific data of microalgae identified in this large area was scarcely reported. This research aimed to isolate, to cultivate and to characterize potential species of freshwater microalgae in term of their growth rate, chlorophylls content, lipids content, and potential for biodiesel production. Moreover, since the conventional solvent extraction and transesterification of microalgal lipid to biodiesel require several steps (Patil et al., 2015 and Chen et al., 2015), then the method needs to simplify to a direct trans-esterification process from wet or dry microalgal biomass.

Material and methods

Materials

Several equipment, tools and chemicals were used in this method such as laminar airflow (GEA model YX-24LDJ), autoclave (Messgerate H915s), sonicator (Brauosonic SPA ITALY), centrifuge, lux meter (Krisbow KW06-288), Gas Cromatoghraphy, Aerator, UV-Vis spectrophotometer, micro pipet (Toppette Pipettor), analytical balance, microscope, Erlenmeyer flask, petri dish, and laboratory glassware. Meanwhile, chemicals used are acetone, chloroform,

methanol, n-hexane, sulfuric acid, urea fertilizer, NPK fertilizer, and agar powder. Sample was isolated from Dok VIII area of Jayapura Papua.

Methods

Sampling was conducted by taking 1-10 liter of liquid sample on location then stored in laboratory. Afterward, it was used for cultivation. All glassware was sterilized by autoclave before cultivation procedure. Agar medium was prepared as follows, each of 3g of agar powder, 0.03g of urea and 0.01g of NPK were dissolved in 500mL of aquadest in Beaker glass. The solution then was moved to Erlenmeyer flask for sterilization procedure using autoclave at 121^{o}C for 20 min. After cooling down the solution then poured to petri dish in laminar airflow. Sample was then streaked to the agar medium and incubated at room temperature. Colonies of microalgae was observed under microscope then identified based on references. The selected colony of microalgae was subsequently moved and cultivated in liquid medium of the same growth medium. Optical density of the culture (OD660nm) was monitored each day using spectrophotometer to make a growth curve.

Chlorophylls were measured based on Arnon method (1949). Ten milliliter of microalgae culture was centrifuged at 4500 rpm. The supernatant was poured by 10 mL of acetone then homogenized for 20 min. The solution was centrifuged again at 4500 rpm for 10 min. Filtrate was used for chlorophylls measurement using UV-Vis spectrophotometer at wavelengths of 645nm (A_{645}) and 663nm (A_{663}). Chlorophylls contents were calculated based on formula below:

Chlorophylls a = $(12.7 \times A_{663}) - (27 \times A_{645})$
Chlorophylls b = $(22.9 \times A_{645}) - (4.7 \times A_{663})$
Total chlorophylls = $(20.2 \times A_{663}) - (8 \times A_{645})$.

Produced biomass harvested at the end of log phase was centrifuged at 4500rpm for 15 min and dried at 60^{o}C to constant weight in vials. As much as 0.2579 g of dried lipid was used in lipid extraction by 4 mL of n-hexane under ultrasonic frequency of 60 KHz at temperature of 60^{o}C for 120 min. Lipid obtained was 0.0784 g or 30% w/w of dried microalgae. Meanwhile, for biodiesel production, lipid in 70 mg of dried microalgal biomass was directly transesterified by adding a mixture of chloroform (0.575mL): methanol (0.425mL) (volumetric ratio of 1.35) in the presence of 50 µL of sulfuric acid (98%) and mixed 5 seconds using vortex then subsequently sonicated at 60^{o}C for 120 min. Afterward, 1 mL of deionized (DI) water was added to the mixture then centrifuged at 4500 rpm for 15 min. The organic phase was then separated well using micro pipette, dried overnight in 60^{o}C oven. Then, the produced microalgal biodiesel was analyzed for its fatty acid composition and percent of FAME using AOAC (2012):969.33 method of gas chromatography as described (Mandik et al, 2015).

Results and Discussion

Colonies of green microalgae (*Chlorophyceae*) were identified in agar medium after 10 days of cultivation by observing the sample under microscope. Small amount of green area was then moved to a fresh broth medium for a sequential cultivation. After 10 days of cultivation there were several colonies of green microalgae observed. The colonies were identified as *Scenedesmus* sp., *Nannochloropsis* sp., *Eudorina elegans*, *Chorella* sp., and *Zoochlorella* sp. as presented in Figure 1. Among the colonies cultivated and observed the *Scenedesmus* sp. exhibited high growth rate compared to other species. Therefore, the *Scenedesmus* sp. was then cultivated in a fresh liquid medium containing urea and NPK fertilizer to produce more biomass using simplified method adapted (Mandotra et al., 2014).

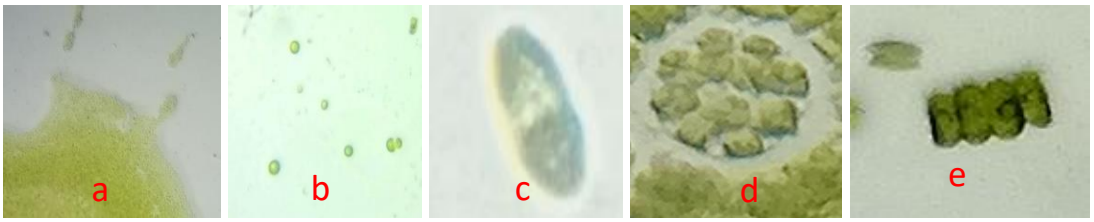

Fig 1. Several green microalgae isolate from Jayapura Papua: a. *Scenedesmus* sp., b. *Nannochloropsis* sp., c. *Eudorina elegans*., d. *Chorella* sp., e. *Zoochorella* sp.

The growth curve of *Scenedesmus* sp. was showed in Figure 2. Lag phase of *Scenedesmus* sp. growth was observed until day 8th. During the lag phase, microalgae adapted to their new environment and growth of microalgae observed was limited. In this phase, protein synthesis and metabolism occurred but cells division has not been happened yet therefore cells density has not increased too. The most growing microalgae biomass observed at the log phase of *Scenedesmus* sp. growth during day 9th to day 16th. Cells division was tremendously improved and growth rate had significantly increased to the optimum conditions. Stationary phase was occurred during day 17th to day 19th followed by dead phase. Meanwhile, this model of growth curve was previously reported by similar studies using *Scenedesmus* sp. (Jena et al., 2012 and Jalal et al., 2013).

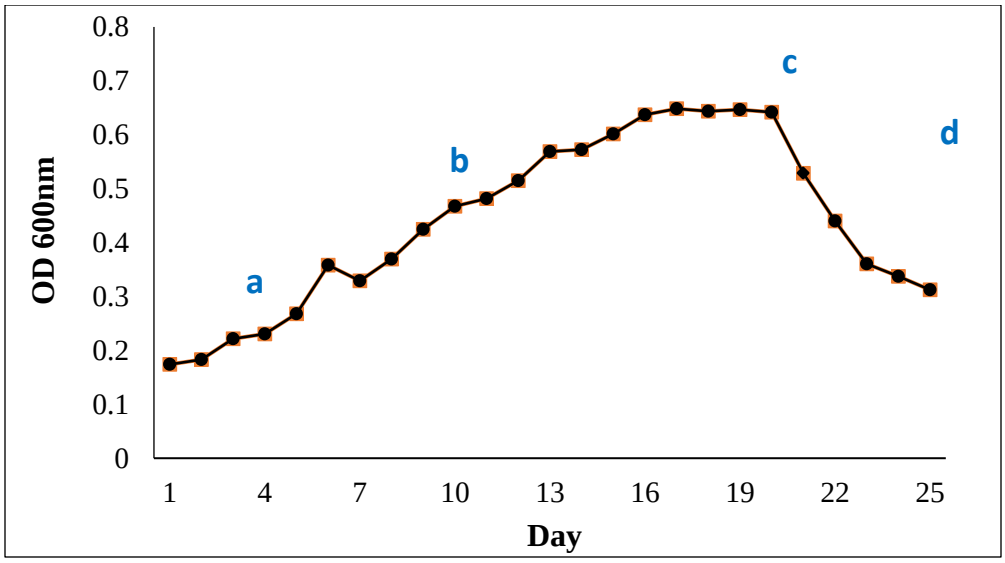

Fig 2. Growth curve of Jayapura isolate *Scenedesmus* sp., light and dark cycle of 12 hours;a (lag phase adaptation), b (log phase growth exponential), c (stasionary phase), and d (death phase).

Chlorophylls content of *Scenedesmus* sp. harvested at the peak of growth rate were 0.4992 mg/L of chlorophylls a and 2.1889 mg/L of chlorophylls b, respectively. Meanwhile, the lipid content was 30% (w/w) slightly higher compared to *Scenedesmus* sp. produced from brewery wastewater reported of 27 % (wt) (Mata et al., 2013).

Figure 3 shows the microalgal biomass and the products after sonication assisted-direct transesterification of microalgal biomass.

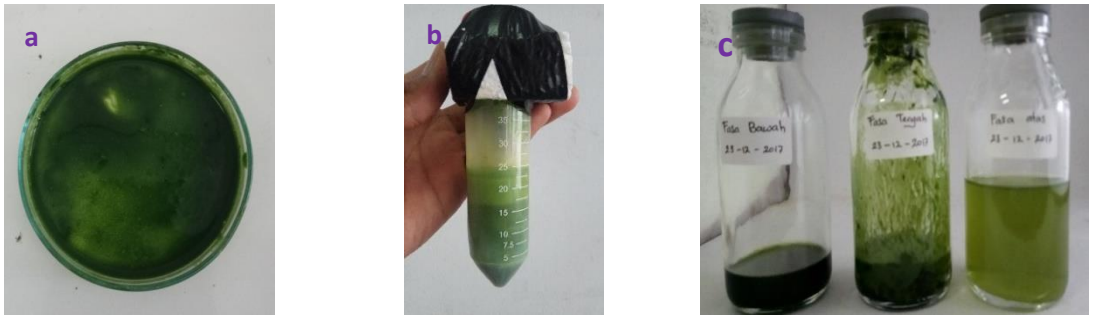

Fig 3. a. Microalgal biomass, b. Product after sonication, and c. Products after separation

Table 1. Fatty acids of biodiesel produced composition from isolated *Scenedesmus* sp

FAME Composition	wt (%)
Palmitic acid (C16:0)	24.21
Linolenic acid (C18:3)	17.09
Linoleic acid (C18:2)	16.52
Stearic acid (C18:0)	12.82
Elaidic acid (C18:1)	5.13
Undefined	24.23
Total	100

Table 1 showed the fatty acids compositions of produced biodiesel. The result was derived based on the comparison between the GC spectrums of sample to the standard of fatty acids. From data obtained, saturated fatty acid named palmitic acid was dominant in the lipid with value of 24.21%, while the highest unsaturated fatty was linolenic acid corresponding to 17.09 %. Other previous studies have reported varied amount of unsaturated and saturated fatty acids obtained corresponding to medium and nutrients used to grow microalgal biomass (Islam et al., 2013; Mata et al., 2013; and Jena et al., 2012).

Conclusions

Jayapura isolate of *Scenedesmus sp.*, exhibits great ability to grow in a cheap fertilizer containing medium compared to other species. The lipids content from microalgae could be utilized as biodiesel feedstock, with some improvements regarding nutrients and separation method. Meanwhile the chlorophylls could be extracted for further uses in health and food industries.

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Community empowerment through facilitation of corn crop farming in Bulude Village, Talaud Islands, North Sulawesi

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Abstract

For the community of Talaud Islands, the agricultural sector, especially food crops, has a strategic and important position not only economically but also for daily survival. However, the food crops business has not contributed significantly to the improvement of people's welfare and the acquisition of healthy, inexpensive, and easy-to-obtain food. Most basic foods such as corn still have to be imported from outside the island. Some of the main problems in the food crops sector in Bulude Village are: 1) low land productivity caused by low knowledge and skill in farming, and also by a moving pattern of agriculture; 2) most of the farming operations are still conventional or traditional as a result of lack of resources of farmers; and 3) motivation and orientation of farming is only to fulfill the daily necessities of life. This article is the documentation of problem solving through community service activities that aim to empower the community through facilitatation of corn crop farming for farmer groups in Bulude Village. The methods of this activity are a combination of the application of appropriate technology, adult learning approach (andragogic), a pilot project strategy, and training and practice field extension techniques. The results of this activity are an increase in knowledge, interest, and skills of the participants in corn crop farming which has direct effects in improving the community welfare both in terms of economic income as well as the need for healthy and quality food.

Key words: Community welfare, food crops.

Introduction

Talaud Islands is the outermost and northernmost region of Indonesia, bordered by the sea with the Philippines (Figure 1). This regency consists of four clusters of islands, namely: Kabaruan, Salibabu, Karakelang, and Nanusa. Based on the Decree of the President of the Republic of Indonesia No. 6, 2017 on the Establishment of the Small Outermost Islands, Kabaruan Island is included in the list along with 110 other islands. Kabaruan Island consists of two subdistricts, namely: Kabaruan and Damau. Kabaruan Subdistrict is bordered by the Karakelang Strait in the North, by the Pacific Ocean in the East, by Damau Subdistrict in the South, and by the Sulawesi Sea in the West. Kabaruan Subdistrict with an area of 115.61 km^2 consists of 12 villages, namely:

Mangaran, Bulude, South Bulude, Kabaruan, East Kabaruan, Kordakel, Pangeran, Pannulan, Pantuge, East Pantuge, Rarange, and Taduna (Central Bureau of Statistics, Talaud Islands Regency, 2015). In general, these villages are classified as underdeveloped villages according to measurable criteria through the *Index Desa Membangun* (IDM, Index of Village Building). This index is a classification system of village status which is a composite of three dimensions, namely: social, economic, and ecological resilience. The classification consists of five village statuses with a score range of 0.27-0.92, i.e.: extremely lagging villages (≤ 0.491), underdeveloped villages (> 0.491 to ≤ 0.599), developing villages (> 0.599 to ≤ 0.707), developed villages > 0.707 to ≤ 0.815), and independent villages (> 0.815). The IDM's third dimension composite score for Bulude Village is 0.592484, so the village is classified as an underdeveloped village (Ministry of Village, Development of Disadvantaged Areas, and Transmigration, 2015).

The geographical position of the Talaud Islands Regency illustrates that in general the district, especially Kabaruan Subdistrict, experiences gaps in various aspects, such as: the level of welfare and quality of life, the availability of facilities and infrastructure, the implementation of good governance, the quality of the environment and food sources, the development of potential in the local economy, and others. The gap becomes more complex when economic activity in this area shifts out of the region. Various food crop commodities have a high potential to be produced here but these areas still import these commodities as well as derivative products from neighboring regions such as Manado and Bitung (Figure 2). For example, coconut crops are plantation commodities that have the widest range of area and the largest number of trees in the district but coconut oil as a derivative product of these commodities is still imported from Manado and Bitung (Capital Investment Coordinating Board, 2011).

Some of the main problems in the agricultural sector in Bulude Village are: 1) poverty due to isolation of areas and limited access to transportation; 2) low exchange rate of agricultural products and agricultural wages; 3) limited facilities for village community infrastructure such as access to electricity for post-harvest processing machines and irrigation channels for irrigation; 4) lack of knowledge, attitude, and skills regarding the management of crop agribusiness; and 5) lack of knowledge, attitude, and skills of farmer groups in processing organic fertilizer by utilizing local potential such as agricultural, livestock, and household waste. To solve these problems various efforts have been made in a program of community service (Science and Technology for the Region/*Ipteks bagi Wilayah* (IbW)) for three years since its implementation started in 2014. Efforts made are community empowerment through facilitation of soybean farming (Maramis et al., 2015), field rice (Maramis et al., 2016a), and vegetables (Maramis et al., 2016b). This article aims to describe the results of community empowerment activities through the facilitation of corn crop farming.

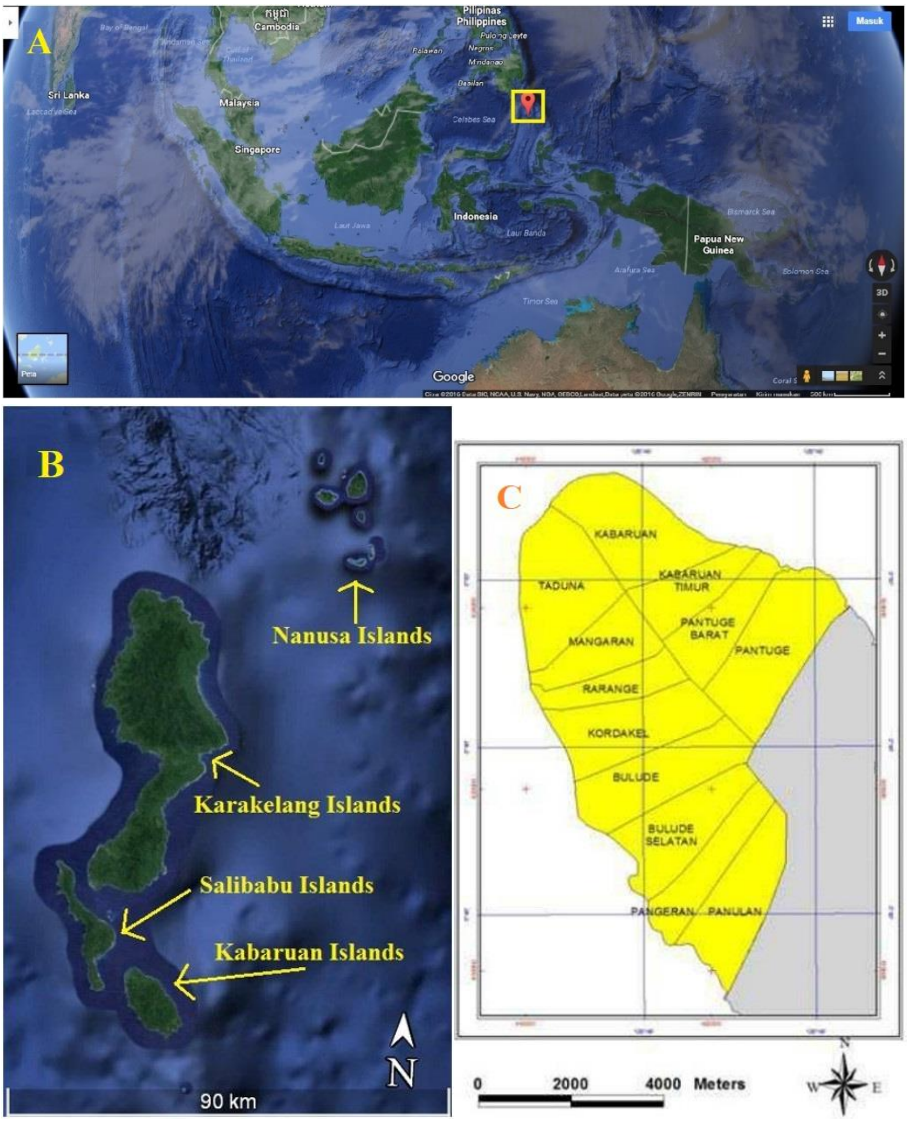

Fig 1. Map of: A) Talaud Islands Regency (yellow box line) in map of Indonesia; B) Kabaruan Islands in Talaud Islands Regency (source: modified from Google Maps, http://google.com/maps, accessed on June 8, 2018); and C) villages in Kabaruan Subdistrict (source: Central Bureau of Statistic, Talaud Islands Regency, 2016).

Fig 2. Basic foodstuffs imported from Manado (source: personal documentation).

Methods

Time and Place of Implementation

Community service activities (IbW schemes) were implemented in Bulude Village, Kabaruan Subdistrict, Talaud Islands Regency, starting from April 2014 until November 2016. Bulude Village was selected because of its high natural resource potential and a chance to become a center of growth and development of a new economy in the region, which is in line with local government plans in making this village one of the pillars of economic buffer.

Problem Mapping and Pilot Project Activities

The initial activities undertaken were problem mapping including surveys and focus group discussions (FGD). After obtaining the results of problem mapping, within the scope of the pilot project activity the method used to overcome the series of community problems was a combination of appropriate applications of technology (Education Office, Central Java Province, 2013) with adult learning techniques, known as andragogic (Suhud, 2005). Meanwhile, the approach to be applied is an approach that departs from the ethics and culture of local communities. Another commonly used approach is Participatory Rural Appraisal and Rapid Rural Appraisal (Chambers, 1994a, b, c) in which issues are fundamentally understandable and revealed and the planning process in overcoming problems is also a common thought with the community itself (Supriatna, 2014). In the implementation of this activity, it has been agreed that what will be done jointly, namely the opening and processing of land, planting and maintenance of corn, and development of agricultural production facilities such as organic fertilizer processing unit based on local resources and postharvest processing unit.

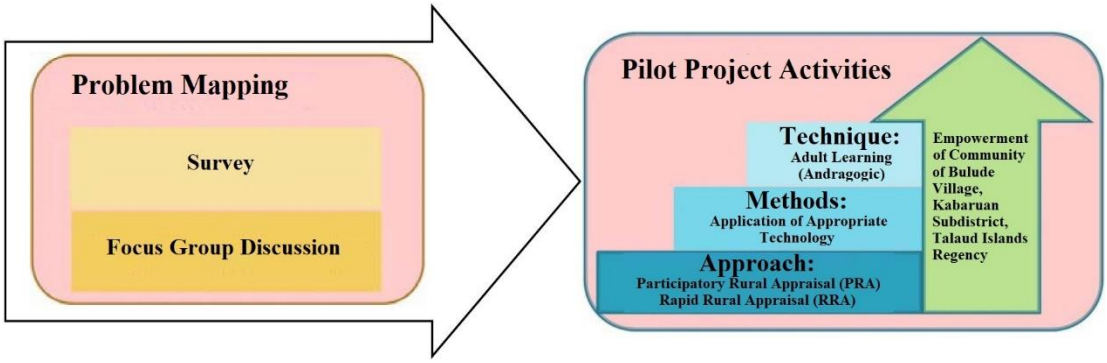

Fig 3. Methods of activity implementation.

Results and Discussion

Corn Crop Farming in Bulude Village

This community service activity began with the socialization of the program to the community and focus group discussion to be more aware of problems faced directly by the community. The activity continued with the preparation of village land on which corn would be planted. Land used to grow corn was abandoned land and land under coconut palm trees. Planting was done by the community and assisted by students who were undergoing field practice activities. Furthermore, the community was trained to make organic fertilizer based on agricultural, livestock, and household waste. Corn crops were not only grown on marginal land and under coconut farms but also on cultivable land such as between vegetable beds to utilize existing land effectively and efficiently. The planting of corn was made gradually with an interval of about 2 weeks in order to maintain the sustainability of production when it is harvested. After reaching the appropriate age the corn is harvested for consumption purposes alone or for sale either in the form of grains of corn or corn rice.

In this activity the community has been guided theoretically about the cultivation of corn crops from the introduction of seeds to postharvest which is concretely done through a pilot project of corn cultivation business covering an area of 10 ha. Corn in the first stage has been planted across 2.5 ha under coconut trees, which serves not only corn production efforts but also overcomes the problem of low productivity of coconut plants. The amount of 9 tons of corn grains or 3.6 tons per ha have been obtained. The second planting area of 2.5 ha utilizing marginal land yielded 9.8 tons or 3.92 tons per ha. The low production for the second planting is generally more due to land that is no longer fertile. The first planting under coconuts, in addition to being influenced by soil fertility, is also influenced by the high level of shade of coconuts. The planting of corn on marginal land on less fertile soil was compromised by heat stress and wind exposure causing lower yield.

Fig 4. Documentation of activities: A) and B) socialization activities; C) marginal land; D) preparation of land for planting; E) planting assisted by students; and F) planting corn under coconut palm trees.

Fig 4 (continued). Documentation of activities: G) the corn seeds used; H) preparation of organic fertilizer; I) and J) corn grown under coconut trees; K) corn grown between beds prepared for vegetable crops; L) corn is grown gradually to maintain the continuity of production.

Lesson Learned from the Activities

The problem of poverty remains a crucial issue in the Talaud Islands Regency which requires serious efforts through comprehensive policies. Therefore, in the effort to overcome the problem of poverty an integration program of empowerment is needed in various sectors, including the agricultural sector. In fact, the agricultural sector is one of the basic sectors that occupy a strategic position and become the trigger for the growth and development of other sectors. This activity has facilitated socialization and training about organic fertilizer processing technology made from agricultural, livestock, and household waste in order to overcome the problem of scarcity and the high price of fertilizers. However, the production of organic fertilizers cannot be conducted continuously because the raw material for fertilizers especially from livestock manure is also not available continuously.

Fig 4 (continued). Documentation of activities: M) corn harvest conducted by the community; N) the transport of crops grown on marginal land through steep trails; O) women separating corn from cobs in a postharvest processing unit; and P) grains of corn are milled to corn rice using a grinding machine.

Through this corn farming business activity, the consumer behavior of the community tends to be transformed towards productivism. Along with ongoing activities, the knowledge, attitudes and skills of the community related to corn cultivation increased through training and field practice. Efforts to improve the welfare of life changed the community from sluggish to proactive. The success of this pilot project activity began to be copied by people from other villages who were initially skeptical and even apathetic. The benefits felt by the farmer groups in these activities are the driving force for other villagers to do the same. In the future, if the corn cultivation is done sustainably it will be the main capital for the achievement of food security at the grassroots level. The activities of community empowerment through various types of business facilitation have been widely reported not only in the agricultural sector but also in livestock. Previously, we have reported community empowerment activities through the facilitation of broiler farming (Mege et al., 2015, 2016a), laying hens (Saerang et al., 2016) and pigs (Mege et al., 2016b). Community empowerment activities not only affect the improvement of knowledge, attitudes and skills as well as economic income of the community but also can improve the competitiveness of production (Palapa et al., 2018a) and the existence of natural resources (Palapa et al., 2018b).

Follow Up Plan

Although this IbW activity has ended, in the future mentoring will continue to be given to the community. The community is taught to be independent in continuing the corn farming that has been pioneered. The community will be directed to the expansion of corn production so that it can be done continuously. So far, the need for fertilizer for agriculture in the Talaud Islands region is still dependent on inorganic fertilizers. In addition to the relatively low level of fertilizer availability, the price of fertilizers is also sometimes not affordable by small scale farmers. In fact, the availability of organic material resources derived from agricultural, livestock, and household waste is quite abundant. Therefore, in the future the community will be accompanied to seek the making of organic fertilizer in larger quantities. These activities give positive results for the community in Bulude Village, namely: 1) the improvement of the welfare of the community through the increase of economic income; 2) the fulfillment of the need for healthy food of high quality; as well as 3) the increase of knowledge, interest and skills related to corn farming.

Acknowledgements

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Effects of Fermentation Duration and Cooking Method on The Chemical Properties and Acceptability of *Growol*

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Abstract

Growol is a local staple food made from cassava that has been processed by spontaneous fermentation, which is achieved by soaking in water for 3-5 days. The fermented cassava is then cooked by steaming before consumption. *Growol* contains the bacteria *Lactobacillus* that can produce lactic acid. Currently, most *growol* producers soak the cassava for 2-3 days to reduce the sourness of *growol*. This causes a decrease in the pro-biotic food potential of *growol* as a small amount of the starch is hydrolyzed into sugar and acid, but with an increase in the amylose content. This increase in amylose produces retrograded starch that functions as resistant starch, although the texture of the *growol* remains hard. The purpose of this study was to modify the cooking method to produce a better preferred *growol* with a high lactic acid bacteria content and increased potential yield of retrograded starch. This research was done with a completely randomized design. The cassava used in this research was the local variety Martapura. Variations in fermentation duration were: 0, 24 and 48 hours, and cooking of the fermented cassava was varied: normal steaming for 15 minutes and two cycles of autoclave cooking at a temperature of 121°C. Moisture content, starch, acid, amylose and the total lactic acid bacteria of the cassava and fermented cassava were determined; *growol* samples were analyzed for their moisture content, acid and the acceptability by hedonic test method. The data were analyzed using SPSS-13 for Windows. The research showed that varying the duration of fermentation affected the fermented-cassava starch content and titratable acidity. The longer the fermentation duration, the lower the starch and amylose content of the fermented cassava, whereas the titratable acidity was increased. Based on the aroma, colour, texture and taste, the most acceptable *growol* was that cooked by steaming for 15 minutes with a fermentation process of 48 hours which resulted in a lactic acid bacteria content of $1\times x10^5$ cfu/g.

Key words: fermented-cassava, cooking, growol, acceptability.

Introduction

Growol was a staple food in the Kulon Progo Regency, Yogyakarta, Indonesia a few decades ago. *Growol* is made from cassava using the following processing stages: peeling, washing, cutting, soaking/fermentation, washing, pressing, enumeration and steaming. Spontaneous fermentation of cassava in *growol* processing takes place over 3-5 days (Anonymous, 2015). Fermentation is part of the processing stage of *growol* that is carried out by soaking the cassava in water for several

days. During cassava fermentation, lactic acid bacteria will grow and produce lactic acid. Putri *et al*. (2012) stated that the lactic acid bacteria types that predominantly grow in *growol* are *Lactobacillus plantarum* and *Lactobacillus rhamnosus*. Wariyah and Sri Luwihana (2015) determined that the total *Lactobacillus* in *growol* was 4.7×10^3 cfu/g. *Growol* has been proven to be effective in preventing diarrhea if consumed daily (Lestari, 2009, Prasetia and Kesetyaningsih, 2014).

Currently, the younger generation in Kulon Progo Regency, especially in Kalirejo village, do not like *growol* because of its sour taste, so to fulfill the desires of consumers *growol* producers have reduced the soaking time to two days. This reduction in soaking time hampers the optimal growth of lactic acid bacteria, thereby decreasing its potential as a pro-biotic. According to FAO/WHO (2002), pro-biotics are living organisms capable of providing beneficial effects on the health of their hosts, when consumed in sufficient quantities. On the other hand, a shorter fermentation time can increase the amylose content of the fermented cassava starch. According to Susilowati *et al.* (2008), the cassava harvested between the ages of 7 - 10 months contain of starch between 14.33% - 35.93% with an amylose content of between 12.37% and 18.91%. During fermentation there would be a breakdown of the starch molecules that could increase amylose (Putri *et al*., 2011). Ogbo and Okafor (2015) showed that in the cassava-based food processing, fermentation and cooking under certain conditions could improve retrograded starch that has the potential to metabolise starch resistance. In banana flour, fermentation to increase amylose can be achieved by soaking for twelve hours and heating at a temperature of 121ºC (Nurhayati, 2011).

The problem is that the *Lactobacillus* bacteria are not resistant to high temperatures. Erdiandini *et al.* (2015) stated that the viability of *Lactobacillus* bacteria decreases with the heating treatment. This condition could lower the sour taste of *growol*. Lactic acid bacteria are amylolytic bacteria. During cassava fermentation two processes occur. These are the enzymatic hydrolysis of carbohydrate substrate (starch) into sugar and conversion of sugar resulted lactic acid (Reddy *et al*., 2008), so decreasing the amount of bacteria would decrease the production of sugar and acid. The objective of this study was to evaluate the effect of varying the duration of fermentation and the cooking method of the fermented cassava on the chemical characteristics (amylose content, starch, titratable acidity) and the lactic acid bacteria resistance in *growol*.

Materials and Methods

Materials

The local cassava used as a raw material for *growol* production was Martapura variety and was purchased from the farmers' market in Sangon village, Kalirejo, Kokap, Kulon Progo, DIY, Indonesia. The cassava was harvested at the age of 12 months and was used no more than two days after harvesting. Chemicals for analysis of the starch content, titratable acidity and amylose, such as: KOH (Merck, 85%), indicator phenolphthalein $C_{20}O_{14}O_4$ (Merck, Darmstadt, 1%), ethanol (Merck, 100%); HCl (Merck, 37%) with pro-analysis qualification and MRS media for total lactic acid bacteria (LAB) analysis came from Oxoid Ltd.

Equipments

The equipment used in this research was: a set of cassava fermentation equipment, autoclave (Pressure sterilizer model 1925X Wisconsin Aluminium Foundry C. Inc. 838 South 16th St. Manitowoc, WI 54220), steamer pan (Bima stainless steel), UV Vis Spectrophotometer (Shimadzu UV mini 1240) for analysis of amylose and starch, balance (OHAUS Pioneer PA214), oven (Memmert DIN 40050 IP 20), vortex (Maxi Mix II TY 37600), sensory testing equipment and glassware for chemical analysis from Pyrex Iwaki (Iwaki glass under LIC).

Research procedure

The fresh cassava was analysed for its water content using the static gravimetric method and for its starch content with the Direct Acid Hydrolysis method (AOAC, 1990), the amylose content was analysed by the colorimetric method (Williams *et al*., 1970), titratable acidity (Apriyantono *et al*., 1989). Processing of *growol* refers to Sutanti *et al.* (2013) and Wariyah and Luwihana (2015). The cassava was peeled and cut into 5 cm lengths, furthermore soaked in water at a cassava/water ratio of 1/3 (w/v) and with variations of soaking/fermentation duration of cassava of: 0 hour (control), 24 hours and 48 hours.

The fermented cassava was analyzed for moisture content, starch, total lactic acid bacteria (LAB) (Fardiaz, 1993 in Hidayat *et al*, 2013) and amylose content. Furthermore, it was cooked using various cooking methods: steaming for 15 minutes (such as is done to make normal *growol*) and cooking using two cycle autoclaves at 121°C for 15 minutes (Ashwar *et al*., 2016). The resulting *growol* samples were analyzed for titratable acidity, moisture content, total (LAB) and acceptability of the *growo,l* which was determined by Hedonic Test (Krammer and Twigg, 1966).

Design experiment

This research used Completely Randomized Design with the factors of fermentation duration and cooking method. The difference between treatments was determined by F test then any significant difference between samples was determined by Duncan's Multiples Range Test (DMRT) that was analyzed by SPSS 13.0.

Results and Discussion

Chemical characteristics of fermented cassava

Table 1. showed the chemical characteristics of fermented cassava. Cassava used for making *growol* has a water content of 60.00% ± 0.49% (wb), starch 49.03% ± 1.68% (db) or about 19.60% ± 0.43% (wb), and amylose 46.95% ± 0.23% (db) or 16.72% ± 0.50% (wb). Susilowati (2008) recorded a cassava starch content of between 14.33% and 35.93% with amylose between 12.37% and 18.91%, but the amount of starch and amylose content was also dependent on the cassava varieties. The major components of cassava are starch and water, which can function as a source of carbohydrates in *growol* as a staple food.

Table 1. The characteristics of cassava and fermented cassava

Cooking method	Fermentation duration (days)	Moisture (% wb)	Titratable acidity (%wb)	Starch (%db)	Amylose (%db)
Fresh cassava	-	60.00±0.49	0.49±0.01	49.03±1.68	46.95±0.23
Steaming	24	58.47±3.95**	0.38±0.01[a*]	75.60±1.41[b*]	48.46±0.70c*
	48	57.49±5.19	0.52±0.08[c]	64.15±0.42[a]	36.12±0.21a
Autoclave two cycles	24	57.77±4.46	0.38±0.02[a]	75.57±6.52[b]	47.83±0.47b
	48	54.59±2.29	0.48±0.06[bc]	65.27±4.60[a]	35.83±1.12a

*Means in a column with similar superscript, not significantly different at α =0.05.
**not significantly different.

After fermentation, the moisture content of the fermented cassavas was 54.59% ± 2.29% - 58.47% ± 3.95% (wb) and not significantly different, the starch was about 64.15% ± 0.42% -75.60% ± 1.41% (db), amylose 35.83% ± 1.12% - 48.46% ± 0.70% (db). The water content of the fermented cassava was lower than the fresh cassava, because before cooking the fermented cassava it was pressed and crushed to reduce water until it reached certain softness. In addition, during washing, the fermented cassava was separated from the fiber component, which resulted in a relatively higher starch content. The length of the fermentation had an effect on the starch content of the fermented cassava. The longer the fermentation the lower the starch content. According to Reddy *et al.* (2008), during fermentation, the starch was hydrolyzed into sugar and then lactic acid. This was shown by increasing the titratable acidity in fermentation for 48 hours. The amylose content of the fermented cassava increased slightly. Putri *et al*. (2011) stated that cassava fermentation for 48 hours at temperature of 30°C resulted in a stable amylose content of the fermented cassava but was still dependent on the type of *Lactobacillus* during fermentation.

Growol characteristics

Growol is fermented cassava (Table 1) that has been treated with two different methods of heating, i.e. normal steaming for 15 minutes and cooking using two cycle autoclaves at 121°C for 15 minutes. The resulting analyses of moisture content and titratable acidity of the *growol* are shown in Table 2, and the total lactic acid bacteria is shown in Table 3.

Table 2. Moisture content and titratable acidity of *growol*

Cooking method	Fermentation duration (days))	Air (% wb)**	Keasaman tertitrasi (%wb)*
Steaming	0	59.70±0.50	0.21±0.03[ab]
	24	60.03±4.17	0.20±0.03[a]
	48	58.01±5.19	0.26±0.06[c]
Autoclave two cycles	0	60.02±1.80	0.23±0.03[ab]
	24	57.52±4.87	0.20±0.03[a]
	48	54.63±0.36	0.22±0.04[ab]

*Means in a column with similar superscript are not significantly different at α =0.05.
** not significantly different.

The results of the analysis showed that the moisture content of *growol* was not significantly different, whereas the titratable acidity was significantly different. *Growol* soaked for 48 hours and cooked by steaming for 15 minutes had the highest titratable acidity. Before steaming, *growol* with fermentation duration of 48 hours had the highest titrated acidity, so was the most acidic *growol*. However, all treatments showed that there were decreases in acidity after cooking. This was probably due to part of the lactic acid evaporating during the heating process. According to Komesu *et al.* (2017), lactic acid stability was affected by the temperature and heating time. The higher the temperature and the longer the heating time, the faster the lactic acid degraded. Steaming treatment for 15 minutes was a mild heating condition compared to cooking using autoclaves two cycles at 121°C, therefore the acidity of *growol* tended to be higher.

Besides acidity, a characteristic of *growol* is the *Lactobacillus* bacteria content. The results indicated that the total lactic acid bacteria (LAB) on the fermented cassava increased with an increase in fermentation duration; the lengthier the time of fermentation, the more the total LAB, both in samples prepared for steaming and for cooking in the autoclave. Putri *et al*. (2012) found that the amount of bacteria after cassava fermentation of 48 hours was about $7.5\times x10^{10}$ cfu/g. However, after cooking no LAB was present in the *growol*, except in *growol* which had been treated with soaking/fermentation for 48 hours and was cooked by steaming. Wariyah and Luwihana (2015) found that in *growol* treated with fermentation for 3 days and then steamed, the total LAB was 4.7×10^3 cfu/g. Cooking conditions greatly affected the total LAB. The LAB could grow well at 37°C. Therefore, the higher the temperatures of LAB and the longer they were sustained, the more the resistance fell.

Table 3. Total Lactic acid bacteria (LAB) for fermented cassava and *growol*

Cooking method	Fermentation duration (days)	Total LAB (cfu/g)	
		Fermented cassava	*Growol*
Fresh cassava	0	5.00×10^5	-
Steaming	24	3.27×10^7	-
	48	4.80×10^8	1.0×10^5
Autoclave two cycles	24	4.90×10^7	-
	48	8.10×10^7	-

*Means those in a column with similar superscript are not significantly different at $\alpha = 0.05$.

Acceptability of growol

The acceptability of *growol* was determined based on preferences for the aroma, colour, texture, taste and overall preferences as shown in Table 4. The acceptability of *growol* showed a significant difference. The aroma, texture, taste and overall preferences of *growol* made from cassava fermented (24 and 48 hours) and cooked by steaming, and a sample made without fermentation (0 hours) which was cooked by using autoclave were not significantly different and were judged to be favourable.

Table 4. Acceptability of *growol**

Cooking method	Fermentation duration (days)	Aroma	Warna	Tekstur	Rasa	Kesukaan keseluruhan
Steaming	0	2.35^{a}	2.90^{b}	2.70^{a}	2.85^{a}	2.75^{a}
	24	2.65^{a}	2.40^{ab}	3.55^{abc}	3.15^{a}	3.10^{a}
	48	2.45^{a}	2.50^{ab}	3.00^{ab}	2.45^{a}	2.60^{a}
Autoclave two cycles	0	2.35^{a}	2.00^{a}	3.00^{ab}	2.85^{a}	2.75^{a}
	24	5.35^{b}	3.70^{c}	4.20^{c}	5.30^{b}	5.05^{b}
	48	5.35^{b}	3.00^{b}	3.85^{bc}	5.25^{b}	5.15^{b}

*Means those in a column with similar superscript are not significantly different at $\alpha = 0.05$.

Whereas two samples from fermented cassava with fermentation times of 24 and 48 hours and cooked by using autoclave were not different and were disliked. The Factors that determined the acceptance of *growol* to the panelists were: smell and taste being rather sour, white colour and chewy texture. In *growol* with 24 hours and 48 hours fermentation treatment and cooking with autoclave, the aroma, colour, texture, taste and overall preferences in categories were less acceptable. This was due to a decrease in the aroma, a brownish color, a bland taste and a very soft texture.

A long heating time and a high temperature (autoclave two cycles and temperature 121°C), caused aromatic substance loss. The colour became brown due to intensive Maillard browning. According to Fennema (1985), Maillard's reaction could be due to a reaction between the amino-protein group and the reduction in sugar. According to Wariyah and Luwihana (2016), a reduction in the sugar of fermented cassava became greater with an increase in fermentation time, Hence the browning effect. The texture of the *growol* was also not favoured because it was too soft. This was due to a higher heating temperature and longer fermentation, which resulted in more hydrolyzed starch and more simple sugars, so the *growol* texture was softer.

Conclusion

The duration of cassava fermentation had a significant effect on the chemical characteristics of fermented cassava. The longer the fermentation, the starch content was decreased, and the titratable acidity was increased, but the amylose remained relatively stable. The duration of fermentation and the method of cooking affected the bacterial resistance and *growol* preferences. Cooking the fermented cassava by steaming resulted in a preferred *growol*, while cooking with autoclave produced *growol* which was not well liked *Growol* made with 48 hours of fermentation and cooked by steaming the fermented cassava still contains LAB, while other treatments did not retain LAB. Based on the aroma, colour, texture and flavor of *growol* and its LAB resistance, cassava fermented for 48 hours and cooked by steaming resulted in an acceptable *growol* with a total LAB 1.0×10^{5} cfu/g.

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Evaluation of Five Different Methods for Biodiesel Production from Marine *Chlorella* sp. Biomass

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Abstract

This study aimed to evaluate and compare five different methods of biodiesel productions from marine *Chlorella* sp. Biomass. Method I is the two-step procedure of lipid extraction using n-hexane and transesterification. Method II is also the two-step procedure but using chloroform and methanol for lipid extraction. Method III is the three-step procedure of chlorophyll removal by acetone followed by lipid extraction and transesterification as same as Method II. Method IV and V are the direct sonication-assisted transesterification procedures using dry and wet microalgal biomass, respectively. Among five methods tested, the extracted lipid yield obtained in Method III was the highest (22.8±4.8%) followed by Method V (22.2±1.5%), Method IV (21.9±0.3%), Method II (15.9±3.3%) and Method I (5.8±0.6%), respectively (Method IV and V were conducted without catalyst addition to calculate the extracted lipid). However, based on GC (gas chromatography) analysis, Method I exhibited the highest percentage of FAME (Fatty acid methyl ester) of 13.18±1.28% followed by Method III (3.66±0.56%), Method IV (3.06±0.05%), Method V (1.55±0.05%) and Method II (1.00±0.21 %). Although Method IV gave lower percentage of FAME and biodiesel yield, this method can reduce step and time in biodiesel production due to the simplify process of extract lipid from dry biomass and followed by direct sonication-assisted transesterification.

Keywords: chlorophyll removal, FAME, GC, marine Chlorella sp., microalgal biodiesel.

Introduction

Microalgae are photosynthetic microorganism which can utilize sunlight and carbon dioxide to produce biomass, and oxygen. The oxygen produced is approximately 50% of total atmospheric oxygen. Microalgal cells develop and grow in water suspension, so in utilizing water, carbon dioxide, and other nutrition, microalgae have higher level of efficiency than plants (Deng *et al*., 2009; Widjaja, 2009). Most species of microalgae produce specific products inside and outside the cells such as, fatty acids, sterol, peptides, polysaccharides, enzyme, carotenoid, antioxidants, and toxins (Hossain *et al*., 2008). Microalgae are also known as microorganism with capability as

biofuel factory. There are several biofuels which were produced from microalgae. They were biodiesel (produced via transesterification reaction), bioethanol (produced via fermentation), hydrogen, and biogas (Basmal, 2008). Comparing to food-based plants/crops, the utilization of microalgae as raw material in biofuel production gives several advantages such as, higher growth rate, higher productivity, utilizing freshwater or sea water, lower water consumption, not compete with food, and relatively low production cost. It is because the simple structure of their cells, and the water suspension environment. Therefore, wider cell surface can capture more light and increase the transfer of mass, resulting faster substrate consumed and more efficient photosynthesis (Miao and Wu, 2006; Sheehan *et al*., 1998).

Lipid accumulation of four strains of microalgae (fresh water *Chlorella* sp., marine *Chlorella* sp., *Nannochloropsis* sp. and *Cheatoceros* sp.) had been studied by Cheirsilp and Torpee (2012). They found that under photoautotrophic cultivation, microalgae produced the highest lipid content compared to those under mixotrophic and heterotrophic cultivation. Marine *Chlorella* sp., *Nannochloropsis* sp., and *Cheatoceros* sp. had the lipid content of about 30% based on dry weight. In contrast, fresh water *Chlorella* sp. had lower lipid content of about 10%. Those results were in line with the studies of Chojnacka and Noworyta (2004) and Liang *et al*. (2009), they reported that the lipid content of *Chlorella vulgaris* which were cultivated under photoautotrophic condition was higher than those cultivated under mixotrophic and heterotrophic conditions. Lipid content of microalgae varies from species to species in range of dry biomass 5 to 77 wt.% (Brown *et al*., 1997; Chisti, 2007). Lv *et al*. (2010) investigated lipid compositions of some microalgal species. They found that some microalgal species are rich in neutral lipids content. The composition and fatty acid profile of microalgal lipids are affected by the microalgal life cycle and the cultivation conditions, such as medium composition, temperature, illumination intensity, ratio of light/dark cycle, and rate of aeration (Guzman *et al*., 2010; Ota *et al*., 2009). Microalgal fatty acids vary from 12 to 22 carbons in length and could be found either as saturated or unsaturated type. The number of double bonds in the fatty acid chains never exceeds 6 and almost all of the unsaturated fatty acids are cis isomers (Medina *et al*., 1998).

There are several important procedures for extracting lipids from microalgae, such as mechanical pressing, milling, homogenization, solvent extraction, ultrasonic-assisted extraction, supercritical fluid extraction, enzymatic extractions, and osmotic shock. Every method has its individual advantages and disadvantages (Mercer and Armenta, 2011). The use of a combination method with sonication method in lipid extraction potentially shows faster extractions and higher yields. The sonication enhances extraction process via cavitation process. The cavitation mechanism can be described as, when ultrasonic waves produce bubbles within the solvent, the bubbles then burst close to the microalgal cell walls. That results shocking waves which trigger the releasing of cells' contents (i.e. lipid) into the solvent (Cravotto *et al*., 2008; Wei *et al*., 2008). In the study of Neto *et al*. (2013), they used microalgal dry biomass (approx. 100mg) to evaluate the lipid extraction methodology regarding a sonication bath as pretreatment cell disruption technique (20 min sonication at room temperature) followed by vortex mixing and n-hexane as solvent (20 mL). The

results showed that the proposed method could obtain a lipid content of 15.5% (% dry weight) for *Chlorella minutissima*, 40.3% for *T. fluviatilis* and 39.5% for *T. pseudonana*.

The two-step method of traditional solvent extraction and transesterification of lipid to biodiesel often requires a large amount of energy because of algae dewatering and grinding into powder. For biodiesel production from dry algae, algae drying consumed 84% of the total energy (Patil *et al.*, 2012). Furthermore, the complex process of the traditional two-step method often requires a total of 0.5–1.5 h for extraction and transesterification. To address the large energy consumption of microalgae dewatering and to simplify the conventional two-step method for biodiesel production, wet microalgal biomass should be used to produce biodiesel directly.

In this research, since marine *Chlorella* sp. was identified as good source of lipid (Cheirsilp and Torpee, 2012), five different new methods in producing of biodiesel from marine *Chlorella* sp. biomass were evaluated and compared based on their extracted lipid yield (%), percent of produced FAME (%), and produced FAME profiles.

Materials and methods

Microalgae strain and growth medium

Marine *Chlorella* sp. was obtained from the National Institute of Coastal Aquaculture, Thailand. The mediums used in this study were BG-11 medium (Cheirsilp and Torpee, 2012). One liter of BG-11 medium contains $NaNO_3$ 1.5 g, $K_2HPO_4 \cdot 3H_2O$ 0.04 g, $KH_2PO_4 \cdot 3H_2O$ 0.2 g, EDTA 0.0005 g, Fe ammonium citrate 0.005 g, citric acid 0.005 g, Na_2CO_3 0.02 g and 1 mL of trace metal solution, pH 7.3. One liter of trace metal solution contains H_3BO_3 2.85 g, $MnCl_2 \cdot 4H_2O$ 1.8 g, $ZnSO_4 \cdot 7H_2O$ 0.02 g, $CuSO_4 \cdot 5H_2O$ 0.08 g, $CoCl_2 \cdot 6H_2O$ 0.08 g and $Na_2MoO_4 \cdot 2H_2O$ 0.05 g.

Cultivation of microalgae

Microalgae strain were pre-cultured in 400 mL of BG-11medium in a 500 mL bottle. The pre-cultures were incubated at 30^{o}C and air-aerated at a flow rate of 0.01 mL/min under a 3,000-lux light intensity with a 16:8 h light and dark cycle for 7 days. Afterward, this culture was used as a seed culture. The batch cultivation of the microalgae was performed by inoculating 10% (v/v) seed culture into each 3 L of BG-11medium in a 3.78 L (1 US gallon) glass bottle. Cultures were incubated at 30^{o}C and air-aerated at a flow rate of 0.01 mL/min. The cultures then were illuminated with a 3,000-lux light intensity with a 16:8 h light and dark cycle for 5 days (Cheirsilp and Torpee, 2012).

Screening of transesterification methods for biodiesel production from microalgae

Five different new methods in producing of biodiesel (fatty acid methyl ester/FAME) from marine *Chlorella* sp. biomass were screened as follows: Method I (modified method of Mexwell, *et al*. (1968) to extract lipid from dry microalgal biomass and then the extracted lipid was transesterified by modified sonication-assisted method of Cheng *et al*. (2013)), Method II (modified method of Bligh and Dyer (1959) to extract lipid from dry microalgal biomass and then the extracted lipid was transesterified by modified sonication-assisted method of Cheng *et al*. (2013)), Method III

(modified method of Archanaa, *et al.* (2012) to extract chlorophyll from dry microalgal biomass followed by modified method of Bligh and Dyer (1968) to extract lipid from dry chlorophyll-extracted microalgal biomass (CEMB) and then the extracted lipid was transesterified by modified sonication-assisted method of Cheng *et al*. (2013)), Method IV (lipid in dry microalgal biomass was simultaneously extracted and transesterified by modified sonication-assisted method of Cheng *et al*. (2013)), and Method V (lipid in wet microalgal biomass was simultaneously extracted and transesterified by modified sonication-assisted method of Cheng *et al*. (2013)).

Determination of the fatty acid composition and percent of FAME in microalgal biodiesel

The biodiesel which were produced from biomass of marine *Chlorella* sp. by five new methods then analyzed for their fatty acid composition and percent of FAME using GC. The methods of EN 14103 (2003) was used in determining the percent of FAME. Fatty acids were identified by comparing their retention times with those of standard ones and calculated as percentage based on their respective peak area using a standard mixture of FAME (Jham et al., 1982). The Gas Chromatograph (7890 Agilent Technologies, USA) equipped with a selected column for biodiesel (length 30 m, 0.32 mm I.D., 0.25 µm film thickness) and a flame ionization detector (FID). The operating conditions were as following: inlet temperature 290^{o}C, oven initial temperature 210^{o}C hold 12 min ramp to 250^{o}C at 20^{o}C/min hold 8 min and detector temperature 300^{o}C.

Results and Discussions

The Effect of Different Methods of Biodiesel Production on the Lipid Yields

Lipid yields from five different methods was depicted in Fig. 1. The extracted lipid yield obtained in Method III was the highest (22.8±4.8%) followed by Method V (22.2±1.5%), Method IV (21.9±0.3%), Method II (15.9±3.3%) and Method I (5.8±0.6%). Simultaneous extraction and transesterification were possible to be applied and this gave higher lipid yield than two-step procedure likely due to the effectiveness of chloroform and methanol volumetric ratio of 1.35 to extract microalgal lipids. The presence of water in mixture of solvent can broader the spectrum of extractable lipids.

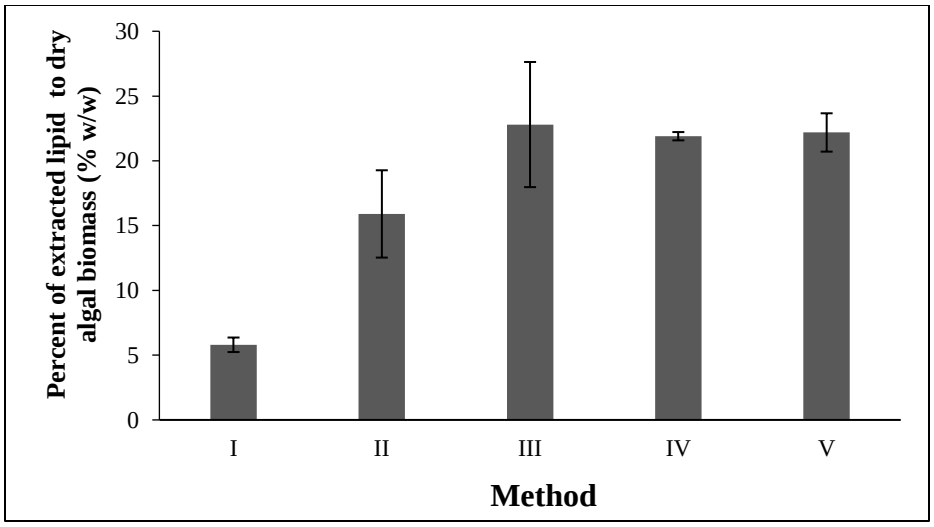

Fig 1. Microalgal lipid extraction by five different methods. Method I: modified method of Mexwell, *et al*. (1968); Method II: modified method of Bligh and Dyer (1959); Method III: modified method of Archanaa, *et al.* (2012); Method IV: modified method of Cheng *et al*. (2013) for lipid in dry microalgal biomass; Method V: modified method of Cheng *et al*. (2013) for lipid in wet microalgal biomass.

The Effect of Different Methods of Biodiesel Production on the percent of FAME

The effect of different methods of biodiesel production on the percentage of FAME can be seen on Fig. 2. Method I exhibited the highest percentage of FAME (13.18±1.28 % based on dried microalgal lipid) followed by Method III (3.66±0.56 %), Method IV (3.06±0.05 %), Method V (1.55±0.05 %) and Method II (1.00±0.21 %). Above results revealed that n-hexane was more suitable for extraction of transesterifiedable compounds. The reaction time (40 min) was too short.

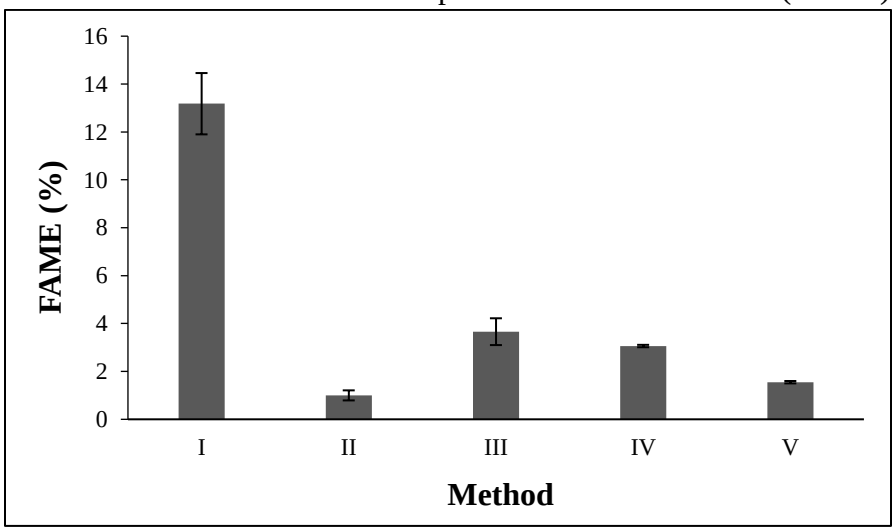

Fig 2. Percent of FAME yield of 5 different methods of biodiesel productions from marine *Chlorella* sp. biomass. Method I: modified method of Mexwell, *et al*. (1968); Method II: modified method of Bligh and Dyer (1959); Method III: modified method of Archanaa, *et al.* (2012); Method IV: modified method of Cheng *et al*. (2013) for lipid in dry microalgal biomass; Method V: modified method of Cheng *et al*. (2013) for lipid in wet microalgal biomass.

The Profile of Fatty Acids from 5 Different Method of Biodiesel Production and Time course data of FAME produced using Method IV

Studying the profile of microalgal biodiesel are normally complicated. It was because, there are many factors that determine their characteristics such as microalgae strain, growth medium, growth conditions, culture mode, type of photobioreactor (PBR), time of harvesting, method of lipid extraction, type of solvent used in lipid extraction, method of biodiesel production, and chosen characterization method. Some of those factors obviously are responsible to the modification or changing the chemical compositions of the microalgal biomass including their fatty acid profiles. Fatty acid profiles measured from the produced biodiesel of 5 different methods using marine *Chlorella* sp. biomass as the feedstock were summarized in Table 1.

Table 1. Fatty acid profiles measured from the produced biodiesel of 5 different methods.

Method	Fatty acid (%)											
	C12	C14	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C22:1	C24:1	USFA	SFA
I	0	0	47.83	2.17	19.57	26.09	4.35	0	0	0	52.17	47.83
II	0	0	25.00	25.00	40.00	5.00	5.00	0	0	0	75.00	25.00
III	0	0	71.06	5.35	2.26	16.27	5.07	0	0	0	28.94	71.06
V	0	0	46.15	3.85	30.77	19.23	0	0	0	0	53.85	46.15
Data of time-course experiment												
IV (40min)	0	0	40.63	6.25	28.13	25.00	0	0	0	0	59.38	40.63
60 min	0.73	6.68	43.32	1.79	5.86	8.96	25.90	5.21	1.55	0	49.27	50.73
120 min	0.88	7.33	42.37	2.12	5.91	8.83	26.92	5.30	0.35	0	49.43	50.57

Table 1 showed that the produced biodiesel by Method I has C16:0 of nearly 50%. Method II produced biodiesel exhibited that C:18:0 was 40%. Method III produced biodiesel showed that C16:0 was 71%. Both Method IV and V produced biodiesels indicated similar composition that C16:0 of around 40% and C18:0 of about 30%. Similar results reported by Fang et al. (2004) and Dayananda *et al*. (2006) who found that palmitic and oleic acids were the main components in *Botryococcus braunii* hydrocarbon content. The marine *Chlorella* sp. fatty acids analyzed in this research show the similarity to the fatty acids of lipid extracted from *Tetraselmis suecica* that had several principal fatty acids such as C16:0, C18:1, and C18:2. Those fatty acids profile were the required fatty acids for conversion to high-quality biodiesel (Halim *et al.*, 2012). These data indicated that marine *Chlorella* sp. lipid has high potential as biodiesel feedstock. Based on time course curve of FAME produced by Method IV (**Fig** 3), it can be seen that after reaction time was prolonged from 40 min to 60 min, this method gave >60% FAME.

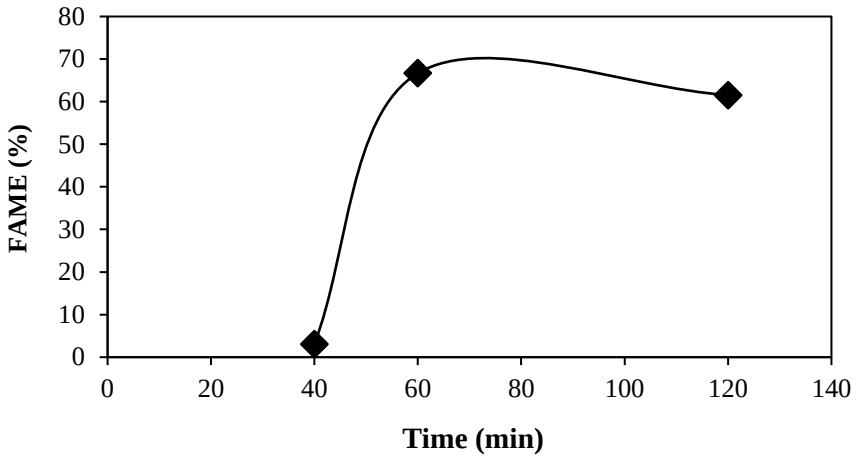

Fig 3. Time course curve of FAME produced using Method IV.

Conclusion

Marine *Chlorella* sp. cells which contain suitable lipids can be directly transesterified to produce biodiesel. The extracted lipid yield obtained in Method III was the highest, followed by Method V, Method IV, Method II, and Method I, respectively. Meanwhile, Method I exhibited the highest percentage of FAME, followed by Method III, Method IV, Method V, and Method II, respectively. Method IV gave comparable lipid yield (21.9±0.3%) and after the reaction time was prolonged from 40 min to 60 min, this method gave >60% FAME. Based on higher FAME obtained by Method IV, this method should be optimized for biodiesel production.

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Improvement of community well-being through programs of non-rice food self-sufficiency in Raanan Baru

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Abstract

Raanan Baru is the capital of West Motoling Subdistrict, South Minahasa Regency, North Sulawesi Province. A community service activity is being implemented in this village with one goal, namely, to improve community well-being in various aspects. Methods of implementation of activities undertaken in accordance with this goal is the empowerment of the community which includes: 1) learning about the technigue of cultivation of taro plants; 2) conducting technical guidance on the way of taro cultivation; and 3) developing pilot project of taro farming business to the partner farmer groups in order to be able to increase the production and ensure the sustainability of production for the market needs. The activities of community empowerment through cultivation learning, technical guidance, and pilot project that have been done give impacts in the form of increasing the economic income of the community so that directly or indirectly can improve various aspects of community values.

Key words: Community empowerment, Taro (Colocasia esculenta (L.) Schott).

Introduction

The village of Raanan Baru consists of three expansion villages, namely: Raanan Baru Village, Raanan Baru Village I, and Raanan Baru Village II; with the total area respectively: 4, 4.5, and 7.5 km^2; the number of inhabitants respectively: 1206 (610 male, 596 female), 953 (486 male, 467 female), and 773 (446 male, 327 female) persons; population density, respectively: 301,5, 211,78, and 103,06 person.$(km^2)^{-1}$; and altitude above sea level respectively: 879, 845, and 892 m (BPS Minahasa Selatan, 2016a). The village of Raanan Baru is one out of eight villages in West Motoling Subdistrict of South Minahasa Regency of North Sulawesi Province, also a district capital. West Motoling Subdistrict has an area of 17,711 Ha (177,11 km^2). The village of Raanan Baru is the smallest village in West Motoling Subdistrict (400 ha) while the largest is Toyopon Village (3580 Ha). West Motoling Subdistrict is located on the slopes of the mountains with an altitude of 462-892 meters above sea level (BPS Minahasa Selatan, 2016b). In West Motoling Subdistrict there are six kindergartens (TK), nine elementary schools (SD), four junior high schools, one high school (SMA), and one vocational high school (SMK). In terms of health facilities, West Motoling Subdistrict has one community health center (*puskesmas*), three sub

community health center (helper *puskesmas*), and eight integrated service post (*posyandu*) (BPS Minahasa Selatan, 2016a).

The people of West Motoling Subdistrict mostly work in agriculture. Geographical conditions are suitable for crops so many people prefer to farm especially in the plantation sector. The largest plantation commodities in this subdistrict are coconut for coconut oil (1,104,02 tons in 2012 and 1,008,24 tons in 2013) and cloves which are the basic ingredients for the manufacture of cigarettes and cooking seasonings, then the palm sugar is the basic ingredients for making brown palm sugar and *cap tikus* (traditional liquor, typical of Minahasa region). West Motoling Subdistrict has potential in the food and beverage industry, especially in processing *cap tikus* and brown palm sugar totaling 365 small industries (BPS Minahasa Selatan, 2016b).

Raanan Baru villagers generally work as farmers with a system of farming is still done traditionally that has been going on for generations. The impact of traditional cropping patterns causes the production or harvest of food products to be relatively low compared to the same crops in other regions. The low production of agricultural products can not yet become the foundation of the people's economy. On the other hand, the agricultural areas of Raanan Baru Village are potential areas to be developed into agricultural production centers of non-rice food crops. This is possible because the agricultural area of Raanan Baru Village is quite extensive, the land is fertile, and is supported by a suitable climate for food crops. The main problem faced by the farmers community in Raanan Baru Village is that they have not realized how important the potential of non-rice cultivation as food source because there is still assumption that the consumption of non-rice food such as taro, cassava and sweet potato is identical with poverty.

This community service activities in Raanan Baru Village is important and very urgent because with the development and advancement of science and technology, gradually non-rice food crops are becoming to be desired by urban households in North Sulawesi. For example, scientific studies have shown that non-rice food crops such as tubers including taro which has a low sugar content, when frequently consumed potentially prevents *diabetes mellitus* (Yalindua, 2014). In addition, survey conducted by Yalindua (2014) shows that non-rice food consumption with various processed products is increasingly reaching the society with middle to upper economic level. Previously, in the same series of programs (*Program Pengembangan Desa Mitra* (PPDM)), it was reported that this activity has been able to improve product competitiveness (Palapa et al., 2018a) and improve the presence of natural resources (Palapa et al. 2018b). This article aims to describe the results of PPDM activities in improving the community's well-being.

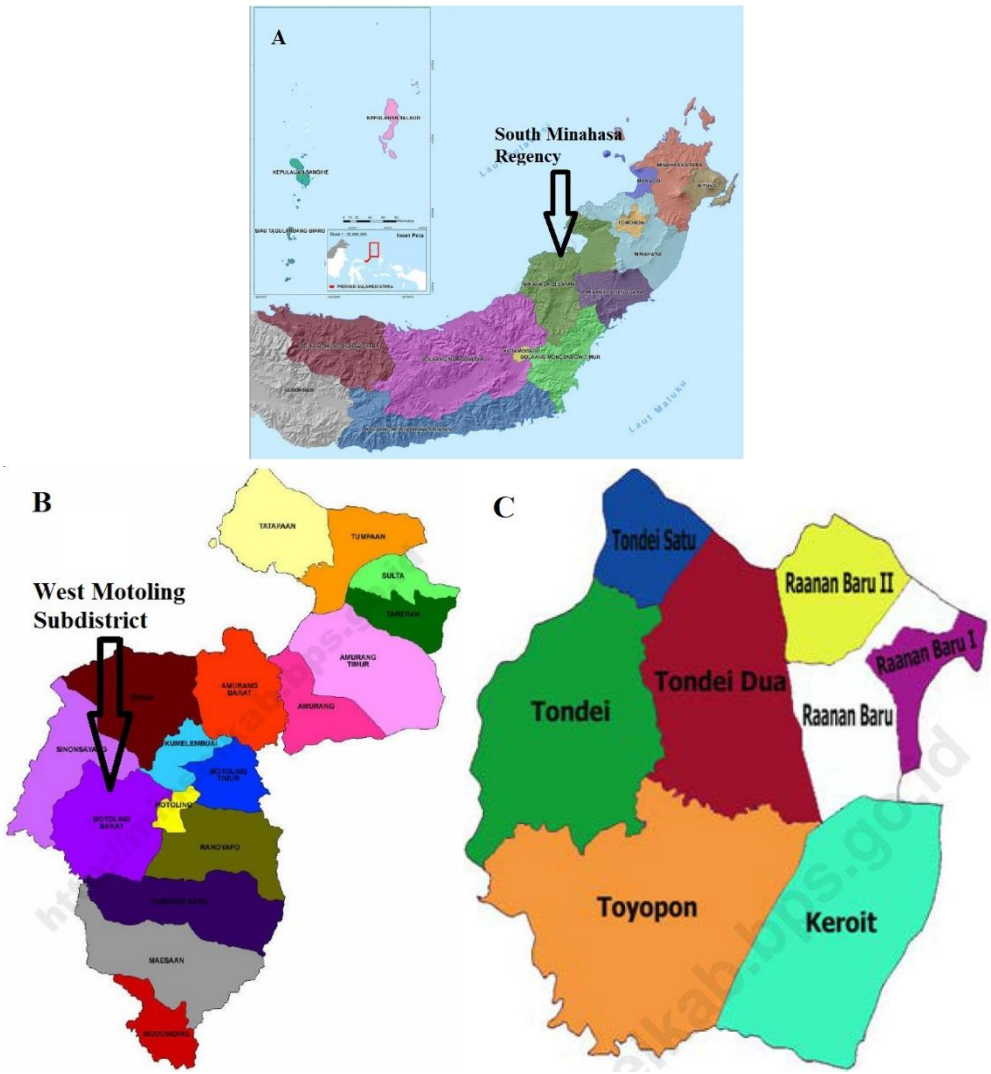

Fig 1. Maps of: (A) Regency in North Sulawesi Province (BPS Minahasa Selatan, 2015); (B) Subdistrict in South Minahasa Regency (BPS Minahasa Selatan, 2017); and (C) Village in West Motoling Subdistrict (BPS Minahasa Selatan, 2016a).

Methods of Implementation

To achieve the objective of activities related to the improvement of community values of Raanan Baru Village, West Motoling Subdistrict, South Minahasa Regency, North Sulawesi Province, the approach of the implementation of the activities is community empowerment through the program of non-rice self-sufficient farming, which includes: 1) techniques of taro cultivation (socialization and counseling); 2) technical guidance and training on non-rice food cultivation; and 3) developing pilot project program of trial garden involving three partner of farmer groups (Solo Kulo, Alfa Omega, and Serentape Farmer Groups).

Results and Discussion

Raanan Baru villagers are largely composed of farmers who tend to rely on crops from perennials. After the activities of IbDM are implemented, then there is a change in behavior patterns that affect the increase in income. This also affects the behavior and values of the society towards social behavior, especially the tendency of association in the form of productive farmer groups, which automatically impact on peace and security. In addition, in every opportunity, the implementing team always conveys the importance of production results directed at improving children's education, with a source of cost from farm produce. Community empowerment through learning, technical guidance, and pilot project program can increase economic income for the community so that directly or indirectly can improve various aspects of community values.

The activity partner consists of 3 farmer groups from partner villages. Each group can support activities because these activities are in direct contact with everyday life. Besides that, farmer groups are more easily coordinated so that the established programs are easier to implement. This is possible because most of the villagers of Raanan Baru are farmers so that they are considered to have the same interests and needs. In addition, this farmer group is an advanced farmer group that has sufficient experience as evidenced by the inaugural letter from the sub-district government. The profiles of the three farmer groups and the conditions before and after the implementation of the PPDM program are presented in Table 1.

Table 1. Profile of farmer group, circumstances before and after PPDM.

No.	Data of partner group	Farmer group		
		Solo Kulo	**Alfa Omega**	**Serentape**
1.	Profile			
	a. Members	30 people	25 people	30 people
	b. Business fields	Cultivation of sweet potato crops	Cultivation of food crops of sweet potatoes, corn and beans	Cultivation of food crops for sweet potatoes, corn, bananas, and vegetables
	c. Efforts made	Increased knowledge about cultivation techniques for sweet potatoes and corn	The working pattern of mutual cooperation, each of which provides land, the processing is carried out in turn	Actively involved in programmed, collaborative activities
2.	Circumstances before PPDM	• Knowledge of cultivation techniques is still traditional • Knowledge of organizational management is still low	• Before applying cultivation techniques, durability of postharvest taro rarely reaches 8 months in dry conditions	Utilizing the land area for coconut and clove plantations

			• Production quantity, 20 kg gross weight of sweet potatoes harvested from 4-5 trees	
3.	Circumstances after PPDM	• Knowledge of cultivation techniques leads to modern agriculture • Improved knowledge of organizational management	• Durability of postharvest taro reaches 8 months in dry conditions • Production quantity, 20 kg gross weight of sweet potatoes harvested from 1-2 trees	Utilizing marginal land and land on hillsides

Fig 2. Documentation related to the improvement of community well-being: (A) The team disseminates in a meeting forum of School Committee of *SMA Negeri 1* (Senior High School) of West Motoling; (B) Parents listening to the socialization of PPDM activities; (C) Teachers involved in socialization; and (D) Teams and member of the School Committee review the land around the school.

Activities carried out through the PPDM program include: 1) techniques for cultivating non-rice food crops; 2) improvement of quality and diversification of product; and 3) introduction to the basics of entrepreneurship. A brief description of the activities carried out is listed in Table 2. Community empowerment activities through various types of business facilitation with similar positive impacts have been widely reported. Call it, as done by Mege and colleagues, through the facilitation of broiler farming business based on local resources in Bulude Village, Kabaruan Subdistrict, Talaud Islands Regency (2015) and in Musi Village, Lirung District, Talaud Islands Regency (2016) as well as business facilitation of a pig farm based on local resource in Bulude Village (2016). Saerang and colleagues (2016) have also done business facilitation of laying hens, so as to overcome the disparity between the production and consumption of chicken eggs in Musi Village. Not only in livestock, Maramis and colleagues through community service programs (Regional Partnership Program/*Program Kemitraan Wilayah* (PKW)) has also undertaken various types facilitation in the field of agriculture based on local resource to improve community welfare in Bulude Village, such as facilitation of soybean farming (2015), facilitation of field rice farming (2016a), and facilitation of vegetable farming (2016b). The results of these PPDM activities and the facts reported in these references reinforce evidence that community empowerment has a positive impact on partner villagers.

Table 2. Activities carried out through the PPDM program

No.	Activities of PPDM	Description of activity
1.	Techniques of non-rice food crop cultivation	
	a. Land processing	Land processing is done to prepare the land until it is ready to be planted. Processing is done by plowing or hoeing and mashing until the soil becomes loose.
	b. Seed preparation and planting	The seeds to be planted have been prepared in advance. Generally, the seeds of food crops are planted directly without being preceded by seeding and most of them are allowed to grow themselves.
	c. Fertilization	Fertilization aims to provide adequate nutrition for plant growth and development.
	d. Plant maintenance	Maintenance activities include refining, watering, and planting. Watering is done to keep the soil moist.
	e. Controlling pest	Pest control is adjusted to the level of attack and carried out manually or with pesticides.
	f. harvest and postharvest	Harvesting is the last stage of food crop cultivation, after that it will enter the postharvest stage.
2.	Improvement of quality and	Generally, non-rice food is only used as a staple food substitute for rice by the people of Raanan Baru Village. This habit can be changed by educating the public on the importance of diversifying processed products of non-rice food.

	diversification of product	To further increase the selling value, the quality of snacks processed by non-rice foodstuffs must be improved as well. Quality improvement can be done on two characteristics, namely: 1) physical characteristics, including appearance (color, size, shape, presence or absence of physical defects), texture, taste; and 2) hidden characteristics, including nutritional value and food safety.
3.	Introduction to the basics of entrepreneurship	In this activity, the village community who are members of the partner group will receive material training in the form of: successful entrepreneurial character, creative and innovative ideas, market research, risk management, production planning and management, finance, and marketing.

Conclusion

This PPDM activity held in Raanan Baru Village, West Motoling Subdistrict, South Minahasa Regency, North Sulawesi Province, which started from April 2017 can take place smoothly. This activity can significantly increase the economic income for the community so that it can directly and indirectly improve community well-being in various aspects such as art and culture, social, politics, security, peace, education and health.

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Investigation of Several Determining Parameters in Flocculation Process of Marine *Chlorella* sp.: A new promising biodiesel feedstock

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Abstract

Screening for a highly efficient method in harvesting microalgae is an important step to a large scale microalgal biodiesel production. Low final concentration of microalgal biomass and small size of microalgae make harvesting of the algal biomass is challenging. Flocculation of microalgal cultures by addition of inorganic compounds under acidic or alkaline conditions is known as the most promising method for harvesting of microalgal biomass. This study aimed to investigate several determining parameters in flocculation process of marine *Chlorella* sp. Magnesium salt ($MgSO_4 \cdot 7H_2O$) concentration of 0.0083 g/L of marine *Chlorella* sp. culture with biomass concentration of 3.78 g/L showed the highest flocculation efficiency (FE) of 94.63% at pH of 11 after only 10 minutes of flocculation time. There was no any difference of FE between two different volumes of culture tested, after 10 minutes of flocculation. Therefore, to flocculate 1000 L of marine *Chlorella* sp. culture with biomass concentration of 3.78 g/L at pH of 11, needs only 10 mins of time by adding 8.3 g of $MgSO_4 \cdot 7H_2O$ to achieve 94.63% flocculation efficiency.

Keywords: *Chu 13, flocculation efficiency, magnesium salt, marine Chlorella sp., photoautotroph, rapid harvesting*

Introduction

Microalgae are more promising biofuel feedstock compare to the previous two generations of energy sources of biofuel, due to the prospect of these organisms which show high biomass yields without requiring any arable land (Chiaramonti, 2007; John *et al*., 2011; Trent, 2012). However, harvesting of microalgal biomass consume large amount of energy. It jeopardizes the massive interests of algal biomass from the result of energy analyses and the life-cycle assessment (Zheng *et al*., 2012).

Low final concentration of microalgal biomass and small size of microalgae make harvesting of the algal biomass is challenging. Flocculation of microalgal cultures by addition of inorganic compounds under acidic or alkaline conditions was known as the most promising method for

harvesting of microalgal biomass. It was because flocculation could be easily scale-up and applied for various species of microalgae (Uduman *et al*., 2010). Therefore, in the view of economic and technological feasibility, flocculation can be a convenient and an effective method for harvesting microalgae from large scale of microalgae cultures (Wu *et al*., 2012).

Since Cheirsilp and Torpee (2012) reported that marine *Chlorella* sp. under photoautotrophic cultivation could accumulate lipid to about 30% based on dry weight and showed promising potentials as biodiesel feedstock. Then this study aimed to investigate several parameters that related to marine *Chlorella* sp.'s harvesting process such as the effects of magnesium salt addition, time of flocculation after the magnesium salt addition, pH before the magnesium salt addition, and volume of marine *Chlorella* sp. photoautotrophic cultures, to the flocculation efficiency (%). Biodiesel produced from chlorophylls-extracted microalgal biomass (CEMB) of marine *Chlorella* sp. was also studied.

Materials and methods

Microalgae strain and growth medium

Marine *Chlorella* sp. was obtained from the National Institute of Coastal Aquaculture, Thailand. The mediums used in this study were modified Chu 13 medium (Largeau et al., 1980). One liter of Chu 13 medium contains 0.4 g KNO_3, 0.08 g K_2HPO_4, 0.107 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.02 g Fe citrate, 0.1 g citric acid, 0.00002 g $CoCl_2$, 0.00572 g H_3BO_3, 0.00362 g $MnCl_2 \cdot 4H_2O$, 0.00044 g $ZnSO_4 \cdot 7H_2O$, 0.00016 g $CuSO_4 \cdot 5H_2O$, 0.000084 g Na_2MoO_4, 1 drop of 0.072 N H_2SO_4 and 1 mL of trace metal solution. The pH was adjusted to 7.8. One liter of trace metal solution contains H_3BO_3 2.85 g, $MnCl_2 \cdot 4H_2O$ 1.8 g, $ZnSO_4 \cdot 7H_2O$ 0.02 g, $CuSO_4 \cdot 5H_2O$ 0.08 g, $CoCl_2 \cdot 6H_2O$ 0.08 g and $Na_2MoO_4 \cdot 2H_2O$ 0.05 g.

Cultivation of microalgae

Microalgae strain was pre-cultured in 400 mL of Chu 13 medium in a 500 mL bottle. The pre-cultures were incubated at 30^{o}C and air-aerated at a flow rate of 0.01 mL/min under a 3,000-lux light intensity with a 16:8 h light and dark cycle set up by time regulator for 7 days (Cheirsilp and Torpee, 2012). This was used as a seed culture. The batch cultivation of the microalgae was performed by inoculating 10% (v/v) seed culture into each 3 L of Chu 13 medium in a 3.78 L (1 US gallon) glass bottle. Cultures were incubated at 30^{o}C and air-aerated at a flow rate of 0.01 mL/min. The cultures then were illuminated with a 3,000-lux light intensity with a 16:8 h light and dark cycle for 5 days. During microalgae cultivation, ten millilitres of sample was taken every day. The optical density at 660nm (OD_{660}) of cultivated microalgae were measured by using spectrophotometer (Libra S22 Biochrom). The pH of the culture was measured every day by using pH meter (Mettler Toledo). The dry mass of microalgae, and the specific growth rate were determined.

Flocculation of the microalgal biomass

Microalgal biomass were harvested by flocculation method using flocculent ($MgSO_4 \cdot 7H_2O$). Flocculation experiments were performed by varying pH of microalgal culture (9.5-12), time of flocculation (10-60min), flocculant's concentration in microalgal culture (1.5-8.3 mg/L), and volume of algal culture (0.02-3.78 L). Flocculation efficiency (FE) was calculated using the following equation (Wu *et al*., 2012):

Flocculation efficiency (%) = (1-A/B) x 100

A is the OD_{660} of supernatant from half the height of the clarified layer after flocculation and B is the initial OD_{660} of the algal culture suspension. Meanwhile, the photos of microalgae before and after flocculation were taken by using light microscope (Nikon, 1000x with oil immersion) equipped with Improved Neubauer Bright Line (BOECO, Germany), and digital camera (Samsung, Korea).

Results and discussions

Flocculation efficiency was measured in condition of without flocculent addition and with flocculent addition by varying the pH of culture (9.5, 10.0, 10.5, 11.0, 11.5, 12.0) and time of flocculation (10, 30, and 60 min). Culture volume used for flocculation was 20 mL with

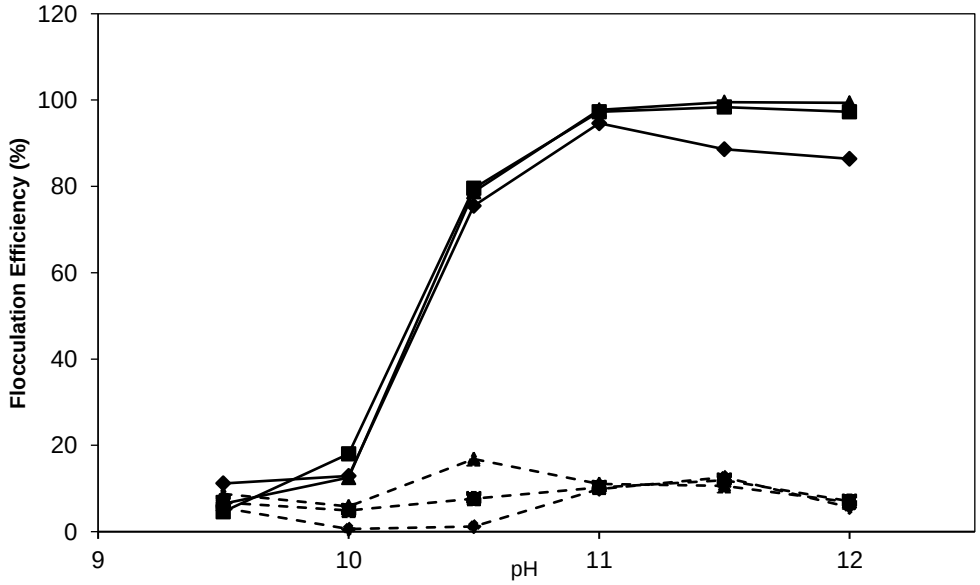

Fig. 1. Flocculation efficiency (%) at various pH and time of flocculations (min) of marine *Chlorella* sp. (solid line represents flocculation with $MgSO_4 \cdot 7H_2O$ of 0.0083 g/L culture; culture volume of 0.02 L; dash line represented flocculation without $MgSO_4 \cdot 7H_2O$; ◆ 10 min, ■ 30 min, ▲ 60 min).

Microalgal concentration of 3.78 g/L, and flocculent ($MgSO_4 \cdot 7H_2O$) concentration of 0.0083 g/L culture suspension which was found as the selected concentration of flocculent. The highest flocculation efficiency (FE) achieved with flocculent addition was 99.7% at pH 12 after 60 minutes while the highest FE achieved without flocculent addition was 20.7% at the pH of 10.5 after 60 minutes (Fig. 1). Result of this study is extremely higher compared to previous study that reported only maximum of 33% of FE when harvesting *Nannochloropsis oculata* for biodiesel production (Surendhiran and Vijay, 2013).

Furthermore, based on t-test: paired two sample for means of FE (%) between two flocculation times (10 min and 30 min) respectively 94.63% and 97.30% with flocculent addition at pH 11. There was no significant difference of FE between those two flocculation times. Therefore, the optimum FE (94.63%) after 10 min of flocculation process was preferable. Moreover, based on t-test: paired two sample for means of FE (%) between two different volume of culture (3.78 L and 0.02 L), at pH 11, flocculent ($MgSO_4 \cdot 7H_2O$) concentration is 0.0083 g/L culture, and after 10 minutes of flocculation, it was found that no difference of FE between those two volumes of cultures. Compared to other studies that reported several types of salts used to improve the flocculation efficiency, in this study $MgSO_4.7H_2O$ was found effectively increase the efficiency to a level of 94.63% at the lower concentration and time (Zhu et al, 2018; Surendhiran and Vijay, 2013). Overall, it is clearly observed that the addition of flocculants at several concentrations and retention times showed great improvement of harvesting microalgal biomass while without the addition of flocculants the maximum FE achieved was only 20.7%.

Meanwhile, **Fig** 2 shows the photos of marine *Chlorella* sp. before flocculation and after flocculation using $MgSO_4 \cdot 7H_2O$.

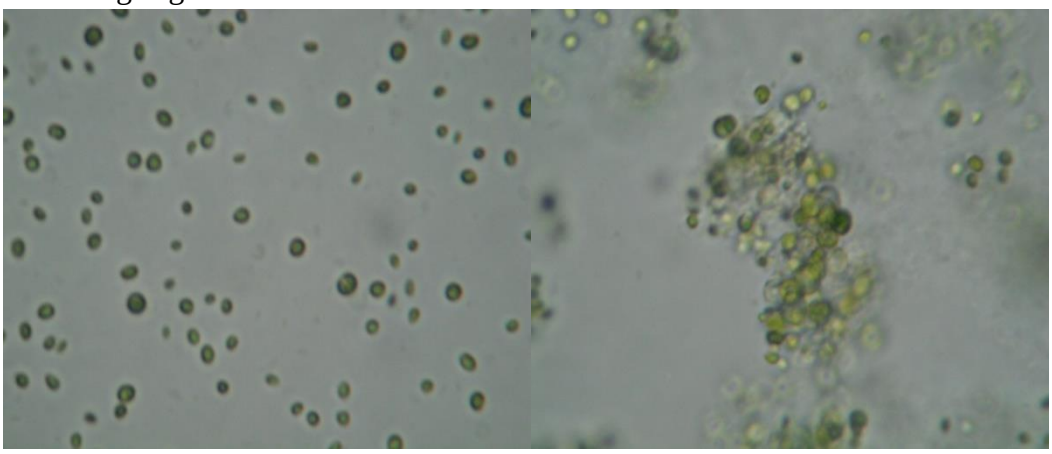

Fig. 2. Photos of marine *Chlorella* sp. were taken. Left: Before flocculation. Right: After flocculation process using $MgSO_4 \cdot 7H_2O$ by light microscope (1000x with oil immersion) equipped with Improved Neubauer Bright Line (BOECO, Germany), and digital camera (Samsung, Korea).

Conclusion

Magnesium salt ($MgSO_4 \cdot 7H_2O$) concentration of 0.0083 gram per litter of marine *Chlorella* sp. culture with biomass concentration of 3.78 g/L showed the highest flocculation efficiency (FE) of 94.63% at pH of 11 after only 10 minutes of flocculation time. Meanwhile, without flocculent addition, the highest FE (20.7%) was achieved at the pH of 10.5 after 60 min. No significant difference of FE (%) was found between two different volumes of culture (3.78 L and 0.02 L) at pH 11, flocculent concentration of 0.0083 g/L culture, and after 10 minutes of flocculation. The $MgSO_4 \cdot 7H_2O$ has displayed great potentials as flocculent in both the efficient harvesting and the rapid harvesting of marine *Chlorella* sp. biomass.

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Isolation and Identification of Antagonistic Yeast From Lemon

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Abstract

Fungal infections are the main cause of post-harvest disease in lemons. A biological agent can treat fungal infection in lemons through an antagonistic interaction. Yeast from lemons is a favorable source of biocontrol agent because they have adapted to the fruit's natural environment. It is the aim of this study to isolate antagonistic yeast that inhibit pathogenic fungus from lemons. Fungus were isolated from rotten store-bought lemons, while yeasts were isolated from fresh and rotten lemons. Based on the morphological observations, the most often occuring fungus having characteristics of *Penicillium* sp. and *Aspergillus* sp. One yeast isolate showed the most powerful antagonistic activity against isolated fungus. Three yeast isolates showed a potentially antagonistic interactions isolated fungus, shown by a growth supression of the fungus.

Keywords: antagonistic yeast, fungi, isolation and identification, lemon, post-harvest disease

Introduction

Microbial communities interact and form relationships among them (Panikov, 2010). Yeasts play an important ecological role in many environments. They are able to strive in nutrient rich ecosystems as well as sustain life in starvation conditions (Muccilli & Retuccia, 2015; Jouhten et al, 2016). Yeasts are commonly found in plants and animals, but they can be found in aquatic ecosystems, soil and fruits (Muccilli & Retuccia, 2015; Perez *et al*, 2016; Salas, 2017). They can benefit from a mutualistic relationship because they produce supporting enzymes or metabolites for its partner and poses adequate metabolic system which is depended on its surroundings (Vega & Blackwell, 2005). With a complex metabolism, the yeast can initiate many forms of interaction amongst yeast or cross-species organisms (Jouhten et al, 2016; Urubschurov et al, 2008; Vega & Blackwell, 2005). Yeasts can create a positive or negative relationship with many symbionts, for example a yeast-insect interaction such as wasp, caterpillar, grasshopper, and fruit fly (Stefanini, 2017; Madden, 2018), yeast-avian interactions (Francesca, 2014), yeast-bacteria interactions (Gibson, 2010; Younis, 2017) and yeast-fungi interactions (Walker et al, 1995; Conti et al, 1998). Antagonism occurs between microorganisms, where one produces a substance or metabolite that changes the environment to create an unfavorable condition that hamper the well-being of another microorganism (Golubev, 2006; Walker et al, 1995). These antagonistic yeasts have been reported to be found in fruits, for example grapefruit, oranges and lemons. Researchers showed antagonistic yeasts that inhibit growth of pathogenic organisms like the fungi (Walker et al, 1995; Perez *et al*, 2017; da Cunha et al, 2018;). It has been reported killer yeasts isolated from lemon with antifungal characteristics against *Penicillium digitatum* and *Penicillium italicum* (Platania et al, 2012; Perez

et al, 2016; da Cunha et al, 2018). These fungi can become pathogenic on fruits that cause post-harvest diseases, which change the physical and chemical appearance of the fruit (Perez *et al*, 2016).

Based on reports, researchers are studying naturally-occurring yeast with the capability of inhibiting fungal growth to manage post-harvest diseases in fruits (Muccilli & Retuccia, 2015). Yeasts have many good traits that suits as biocontrol agent, these include simple nutrient requirements and they can dominate low humidity surface areas and are suitable to survive in bioreactors (Muccilli & Retuccia, 2015). It has been reported yeasts with the capability to suppress the growth of fungi responsible for a common post-harvest disease in lemons (Platania et al, 2012; Perez et al, 2016). The aim of this research is to isolate and identify antagonistic yeast as a biological agent to suppress the growth of pathogenic fungi from lemons.

Materials and Methods

Sample collection

Utilized samples were randomly picked fresh and rotten store-bought lemons from a local supermarket in Yogyakarta, Indonesia. Samples were placed in plastic zipper bags for further analysis. These bags were stored cold before use.

Isolation

Yeast samples were obtained from whole fresh and rotten lemon and were prepared in the same method. Uncut lemons were placed in a 1 L sterile beaker containing sterile water. It was then stirred for about 5 minutes on a magnetic stirrer. A sample of the suspension was taken, diluted up to 10^5, plated on to YPD (Yeast extract 10 $mg.L^{-1}$, peptone 20 $mg.L^{-1}$, dextrose/glucose 20 $mg.L^{-1}$, agar 18 $mg.L^{-1}$ extract) with double antibiotics. Chloramphenicol and tetracycline were used in each working concentration of 25$\mu g.mL^{-1}$ and 10$\mu g.mL^{-1}$ to prevent bacterial contaminants. Inoculated plates were incubated at 28°C for 24-48 hours.

Fungus were obtained from rotten lemon, wash and directly inoculated on PDA as described by (Perez et al, 2016 with modifications). Inoculated plates were incubated at 28°C for 24-48 hours.

Macroscopic identification

Suspected yeast colonies were selected and purified on YPD and incubated at 28°C for 24-48 hours. Single colonies were taken and transferred to new YPD for further analysis. Suspected fungal colonies were transferred to new PDA for microscopic morphology identification.

Microscopic identification

Characterization of yeast isolates were prepared with a drop of yeast suspension on an object glass and was observed under a microscope at magnification 400x. Fungal isolates were prepared through a hanging-drop method with PDA to cellular fungal structures. Characterization was done based on identification key from Samson & Hoekstra (1989).

Antagonistic assay

Eclipse assay was done for screening the antagonistic yeast against fungus. The PDA was used for the test with chloramphenicol and tetracycline with concentrations as mentioned previously. A spore suspension and yeast suspension with 0,1% Tween80 were made from overnight-grown samples. Suspension density was adjusted to 10^6-10^8 cells.mL^{-1} with a haemocytometer. A 20 µL drop of spore suspension was placed in the middle of the solid media and left to dry completely before dropping 2 µL of yeast suspension near the edge of the previously dropped spore suspension. After all drops have dried completely, plates were incubated at 30°C until a fungal growth inhibition was visible.

Results and Discussion

Isoation

Fresh and rotten lemons were sampled to obtain yeast and fungal collection (**Fig** 1). Utilized fresh lemons had a clean, solid surface with a strong lemon scent. Selected rotten lemons look mushy, turns brown, wrinkled, and had a bad rotten smell. Lemon deterioration is commonly caused by a fungal infection (Deacon, 2006). Yeast and fungi have been successfully isolated from citrus fruits (Platania et al, 2012; Perez et al, 2016). These microorganisms are also associated to be present on leaves and soil around the citrus plant (Abdel-sater, 2016; Perez et al, 2016). Several researchers have tested yeast-fungal interactions isolated from fruits and their surroundings. Previous publications have shown existence of an antagonistic yeast isolated from citrus (Taqarort, 2008; Platania et al, 2012; Perez et al, 2016). All isolates were macroscopically identified and resulted in total of 5 potentially antagonistic yeast isolates and 16 potentially lemon-pathogenic fungal isolates. All isolates were randomly picked based on its morphology and dominance.

Fig 1. Appearance of a fresh lemon sample with a clean, shiny and solid surface (without coats) (left) and a rotten lemon with a suspected fungal infection that coats the fruit with a grey-white color (right).

Macroscopic and microscopic characterization of yeast isolates

Yeast colonies were picked randomly, according to its color and shape. Yeast isolates were coded AY1, AY2, AY3, AY4, and AY5. About 150 yeast colonies were obtained, but only five were picked randomly to be analyzed and tested. Their macroscopic and microscopic characters are listed on Table 1.

Yeast colonies were white colored and circular. The colony margin and elevation were smooth, with a shiny surface and produce a sweet scent. Under the microscope, all yeast isolates were multilateral, with yeasts capable of forming up to 4 buds at once. There were three cell shapes from selected yeast isolates; i.e. spheroid, lemon-shaped and ellipsoid (**Fig** 2). All yeast isolates

share many similar characteristics, but they have to be molecularly identified to fully differentiate between them.

Table 1. Cellular and colony morphology of yeast isolates.AY1-5 = Antagonistic Yeast 1 - 5

yeast code	macroscopic characteristics	microscopic characteristics
AY1	white, circular, smooth elevation, shiny	multilateral budding, elongated ellipsoid
AY2	white, circular, smooth elevation, shiny	multilateral budding, lemon-shaped
AY3	white, circular, smooth elevation, shiny	multilateral budding, ellipsoid
AY4	white, circular, smooth elevation	multilateral budding, spheroid
AY5	white, circular, smooth elevation	multilateral budding, lemon-shaped

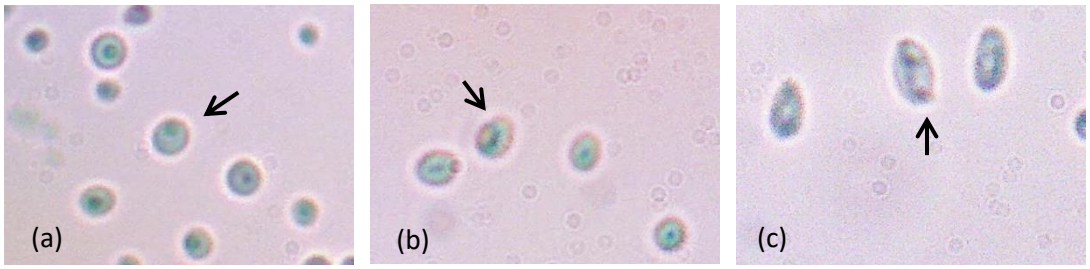

Fig 2. Yeast isolates were microscopicly observed and three different cell shapes were seen. These shapes are shown by arrows (a) a spheroid cell, (b) Lemon-shaped cell and (c) an ellipsoid cell.

Macroscopic and microscopic characterization of fungal isolates

Fungal isolates were characterized according to its macroscopic and microscopic morphology (Table 2). Isolated yeast colonies were macroscopically observed and show similar characteristics i.g. circular, white colored, and smooth elevated colonies with a shiny surface on some isolates. All yeast isolates had multilateral budding. Cellular structure of yeast isolates was ellipsoid, elongated ellipsoid, spheroid and lemon-shaped. These are the characterization keys for yeast isolates. Fungal isolates were categorized into 5 groups i.e. dark green (DG), green (G), grey-green (GG), Black spore (BS), and Green spore (GS) (**Fig** 3). Each fungal isolate was microscopically analyzed and characterized by looking at its branching, spore arrangement. Based on their microscopic morphology, these isolates were suspected as *Penicillium* sp. and *Aspergillus* sp. because they show a close resemblance to the genus (**Fig** 3). These fungi have been reported to be common in microbial communities on decaying environments (Deacon, 2006; Muccilli & Retuccia, 2015; Perez et al, 2016; da Cunha et al, 2018; Dukare et al, 2018).

Table 2. Fungal isolates were observed and characterized based on colony color, reverse color and texture. Most isolates were green colored, with a white reverse, and a powdery or velvety texture. Microscopic observation was based on their branching type, stipe texture, phialides shape. These isolates show different characteristics but share the same typical structure of a specific fungal genus. All DGs, Gs and several GGs isolates were suspected as *Penicillium*, while GS and BS isolates were suspected as *Aspergillus*.

fungal code	macroscopic characteristic	microscopic characteristics	suspected genus
DG1	dark green conidial color, white-yellow edges, yellow reverse, velvety, powdery	simple branching, smooth walled stipe, flask shaped phialides	*Penicillium* sp.
DG2	dark green conidial color white-yellow edges, yellow reverse, velvety, powdery	one stage and two stage branching, smooth walled stipe, flask shaped phialides	*Penicillium* sp.
DG4	dark green conidial color, white edges, yellow reverse, velvety, powdery	one stage branching, smooth walled stipe, short flask shaped phialides	*Penicillium* sp.
DG5	dark green conidial color green-yellow edges, yellow edges, velvety powdery	two stage up to quarter branching, smooth walled stipe, short and long flask shaped phialides	*Penicillium* sp.
G1	green conidial color, white reverse, clear exudate droplet forming, slightly powdery,	simple up to two stage branching, smooth walled stipe, flask globular and elliptical phialides	*Penicillium* sp.
G2	green conidial color, yellow reverse, clear exudate droplet forming, slightly powdery	multistage branching, smooth walled stipe, short flask shaped phialides	*Penicillium* sp.
G4	green conidial color, yellow reverse, small tuft of fasciculate conidiophores but soon smoothes and turns velvety, slightly powdery	simple up to two stage branching, rough walled stipe, smooth walled metulae	*Penicillium* sp.
G5	green conidial color, yellow reverse, velvety, powdery	two stage branching, rough walled stipe, flask shaped phialides	*Penicillium* sp.
GG1	grey-green conidial color, white reverse, powdery	spores group and emerge from branching and stipe tips	ND
GG2	grey-green conidial color, white reverse, powdery	spores group and emerge from branching and stipe tips	ND
GG3	grey-green conidial color white reverse, powdery,	simple branching, smooth walled stipe, Paecilomyces-type phialides, some occurring wavy stipe,	*Penicillium* sp.
GG4	grey-green conidial color white reverse, powdery,	simple branching, smooth walled stipe, flask globular to elliptical phialides	*Penicillium* sp.
GG5	grey-green conidial color, white reverse, powdery,	simple branching, smooth walled stipe, elongated flask shaped phialides	*Penicillium* sp.
GG6	grey-green conidial color, white reverse, powdery	spores group and emerge from branching and stipe tips	ND
GS1	green-white conidial color, white spores, highly elevated,	rough walled, irregular spore formation	*Aspergillus* sp.
BS1	white conidial color, black spores, elevated,	smooth walled stipe,	*Aspergillus* sp.

ND: not determined

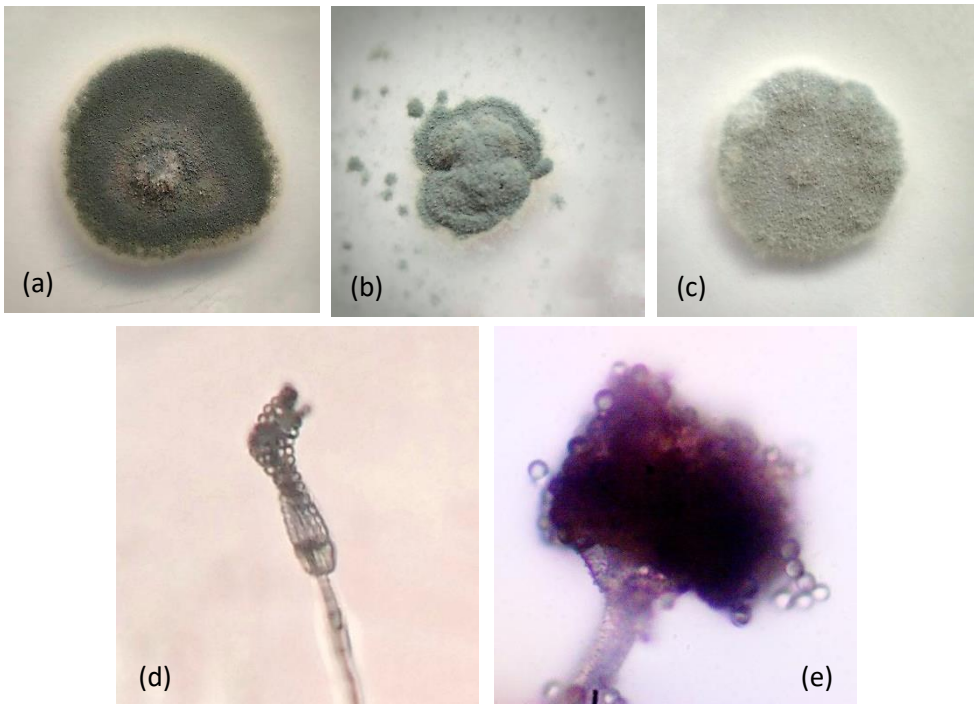

Fig 3. Macroscopic morphology of fungus isolate DG4 (a), fungus isolate G4 (b), and fungus isolates GG4 (C) on PDA, incubated at 30°C for 48 hours. All fungus colonies show different pattern and texture colony, while sharing the same typical microscopic shape from two genus. Microscopic morphology of fungal isoaltes were suspected from *Penicillium* sp. (d) and *Aspergillus* sp. (e)

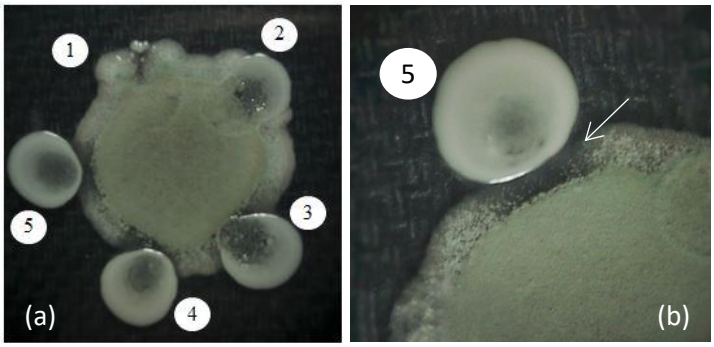

Fig 4. Eclipse assay of yeast isolates AY1-AY5 shown by number 1-5 against fungi isolate DG4 (a). A detailed picture of an inhibition zone (arrow) on isolate DG4 caused by an interaction with yeast isolate AY5 (b).

Antagonistic assay

A growth inhibiton or clear zone is an indication of antagonistic relationship. Yeasts have been known to have inhibition capabilities against fungal cultures (Talibi, 2014; Perez et al, 2016; Al-Qaysi et al, 2017; da Cunha, 2018). Based on this assay, two most common occurring fungal isolates were inhibited by yeast isolates, especially fungi isolate DG4. Fungal isolate DG4 is a suspected *Penicillium*. It was fungus frequently found in lemon samples and was able to be suppressed by three yeast isolates, namely AY3, AY4 and AY5 (**Fig** 4). *Penicillium* sp. is a common fungus found in rotten lemon (Liu et al, 2013; Perez et al, 2016; Perez et al, 2017; Dukare

et al, 2018). *Penicillium italicum* and *Penicillium digitatum* are common fungal pathogens to lemon (Platania et al, 2012; Perez et al, 2016).

Amongst all the yeast isolates, one was able to maintain a consistent inhibition isolate DG4 compared to other yeasts. Isolate AY5 was the most effective competitor that could supress micelial growth of DG4 (**Fig** 4). Two fungal isolates, i.e. isolate DG4 and isolate DG5 were inhibited by this yeast isolate. Isolate DG4 showed constant growth supression compared to other fungal isolates after a double replicate test. A visible clear zone is formed even after 5 days, as seen on **Fig** 4. Investigation based on these findings, AY5 and DG4 deserved to be further analyzed on its antagonistic mechanism and identified molecularly. Understanding the mode of action by the antagonistic yeast can enhance data and give a deeper sense of comprehenssion to the matter.

Conclusion

This research has successfully found yeast isolates that were capable of showing inhibition activities against pathogenic fungi from rotten lemons. A yeast isolate was able to inhibit growth of a fungal isolate. Each of the yeast and fungal isolate were obtained from lemons, which mean they share a similar ecological community and condition but show an antagonistic relationship when interacted together.

Acknowledgement

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Isolation and Identification of Freshwater Microalgae from Jayapura Papua, and Its Utilization in Biodiesel Production using Direct Sonication-assisted Transesterification Method

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Abstract

This study aimed to isolate and identify several lipid-rich freshwater microalgae from small pond in Jayapura Papua. Isolated microalgae were cultivated in fertilizer containing-agar gel medium for identification of microalgae species. Furthermore, the selected species was moved to fertilizer-containing freshwater medium in order to generate large amount of microalgal biomass. The microalgal biomass was then used in biodiesel production using direct sonication-assisted transesterification methods. Several species of microalgae were isolated and identified as *Scenedesmus* sp., *Zoochlorella* sp., and *Eudorina elegans*. Furthermore, *Scenedesmus* sp., which rapidly grew and was the largest part among other species then separately cultivated. Dried biomass of *Scenedesmus sp*. which has lipid content of 30 % was then used in biodiesel production using direct sonication-assisted transesterification method with the sonication frequency of 60 kHz, reaction temperature of 70^{o}C, chloroform: methanol volumetric ratio of 1.35 and reaction time of 120 min. The biodiesel produced has showed composition of palmitic acid (24.21%), linolenic acid (17.09%), linoleic acid (16.52%), stearic acid (12.82%), and elaidic acid (5.13%), respectively. This result exhibited great potential of Jayapura isolate *Scenedesmus sp*., in production of microalgal biodiesel.

Keywords: biodiesel, direct sonication-assisted transesterification, Eudorina elegans, Scenedesmus sp., Zoochlorella sp.

Introduction

Biodiesel was known as one of the important energy resources which is sustainable, environmentally friendly, and economically potentials (Cadenas and Cabezndo, 1998; Sheehan, *et al*., 1998; Antolin *et al*., 2002; Vicente *et al*., 2004; Mandik *et al.* 2015). Several properties and characteristics of biodiesel are also similar to petroleum diesel's (fossil fuel). These similar

characteristics are including, the cetane number, flash point, higher heating value (HHV), and kinematic viscosity (Fuls *et al*., 1984; Knothe., 2010). Biodiesel could also be produced from microalgal lipid through transesterification reaction (Basmal, 2008; Leung *et al*., 2010).
Microalgae, the photosynthetic microorganisms have ability to capture carbon dioxide (as the carbon source) to produce biomass and oxygen by utilizing photon from the sunlight (Deng *et al*., 2009; Widjaja, 2009; Caffarri *et al*., 2014). Microalgae can grow faster than higher plants because their cells structures are simpler, and their environment is mostly a water suspension. Therefore, wider cell surface can capture more light and increase the transfer of mass, resulting in a faster substrate utilization and more efficient photosynthesis (Miao and Wu, 2006; Sheehan *et al*., 1998). It was known that about 50% of total atmospheric oxygen is produced by microalgae (Deng *et al*., 2009; Widjaja, 2009).

During microalgae growth, lipids were synthesized and accumulated in microalgal biomass. Lipid content of microalgae varies from species to species in the range of 5 to 77 % of dry biomass (Brown *et al*., 1997; Chisti, 2007; Mandik *et al.* 2015; Abomohra *et al*., 2016). There are several factors which affect fatty acid compositions of microalgal lipids. The factors are microalgal life cycle, environmental temperature, medium composition, rate of aeration, illumination intensity, and ratio of light/dark cycle (Guzman *et al*., 2010; Ota *et al*., 2009; Ramadan *et al*., 2008).
Papua province of Indonesia predictably has abundance of marine and freshwater microorganism due to its huge tropical biodiversity. However, the reported scientific data of microalgae identified in this large area was scarce. This research was conducted to isolate, to cultivate and to characterize potential species of freshwater microalgae from Jayapura Papua, as Jayapura located close to equator therefore mostly exposes to sun light along year. Moreover, since the conventional solvent extraction and transesterification of microalgal lipid to biodiesel often consumes a large amount of energy and time (Patil *et al.*, 2012; Chen *et al*., 2015), then it needs to simplify the method by using a direct transesterification process from wet or dry microalgal biomass.

Material and Methods

Materials

Several equipment, tools and chemicals were used in this method such as laminar airflow (GEA model YX-24LDJ), autoclave (Messgerate H915s), sonicator (Brauosonic SPA ITALY), centrifuge, lux meter (Krisbow KW06-288), Gas Cromatoghraphy, Aerator, UV-Vis spectrophotometer, micro pipet (Toppette Pipettor), analytical balance, microscope, Erlenmeyer flask, petri dish, and laboratory glasswares. Meanwhile, chemicals used are chloroform, methanol, n-hexane, sulfuric acid, urea fertilizer, NPK fertilizer, and agar powder. Sample was isolated from Dok VIII area of Jayapura Papua.

Methods

Sampling was conducted by taking 1-10 liter of liquid sample on location then stored in laboratory. Afterward, it was used for cultivation. All glassware was sterilized by autoclave before cultivation procedure. Agar medium was prepared as follows, each of 3 g of agar powder, 0.03g of urea and

0.01g of NPK were dissolved in 500mL of aquadest in beaker glass. The solution then sterilized by autoclaving at 121^{o}C for 20 min. After cooling down the solution then poured to petri dish aseptically. Sample was then streaked onto the agar medium and incubated at room temperature. Colonies of microalgae was observed under microscope then identified based on references. The selected colony of microalgae was subsequently cultivated in liquid medium of the same growth medium. Optical density of the culture (OD600nm) was monitored each day using spectrophotometer to make a growth curve.

Microalgal biomass was harvested by centrifugation at 4500rpm for 15 min. Lipid content of microalgae was extracted from biomass using n hexane method of extractions which assisted by sonication at 60kHz and temperature of 60^{o}C for 120 min. Harvested microalgal biomass was directly transesterified by adding the mixture of chloroform (0,575 mL): metanol (0,425 mL) with volumetric ratio of 1,35. Furthermore, 50µL of sulfuric acid (98%) was added then mixed for 5 min. The mixture solution was subsequently heated at 60^{o}C for 120 min. After centrifugation at 4500 rpm for 15 min, the biodiesel layer was separated and used in biodiesel characterization by gas chromatography method for percent of fatty acid methyl esther (FAME) produced (Mandik, 2015).

Results and Discussion

Colonies of green microalgae (*Chlorophyceae*) were identified in agar medium after 10 days of cultivation by observing the sample under microscope. Small amount of green area was then moved to a fresh broth agar medium for a sequential cultivation. After 10 days of cultivation there were several colonies of green microalgae observed. The colonies were identified as *Zoochlorella sp., Scennedesmus* dan *Eudorina elegans* (Table 1). Among the colonies cultivated and observed the *Scenedesmus* sp. exhibited high growth rate and more suitable to grow in the medium. Therefore, the *Scenedesmus* sp., was then cultivated in a fresh liquid medium to produce more biomass. Fig 1 shows the photos of liquid medium which used before inoculation and after inoculation of isolate microalgae, respectively. *Scenedesmus* sp. was selected and the isolate was further cultivated in liquid medium contained urea and NPK fertilizer. The growth curve of *Scenedesmus* sp. was shown in Fig 2.

Table 1. Several microalgae that isolated and identified in this research

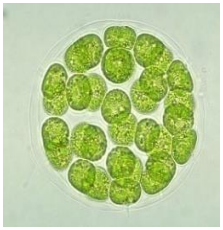
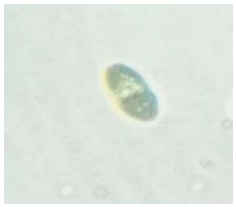
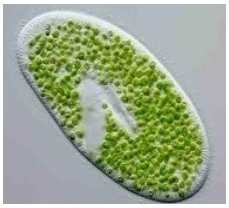
Species	Photo of the isolated microalgae	Photo of reference microalgae	Source of reference
Scenedesmus sp.			cfb.unh.edu
Eudorina elegans			protist.i.hosei.ac.jp
Zoochorella sp.			cfb.unh.edu

Fig 1. Liquid medium for microalgal cultivation. Left: before inoculation. Right: after 10 days of inoculation

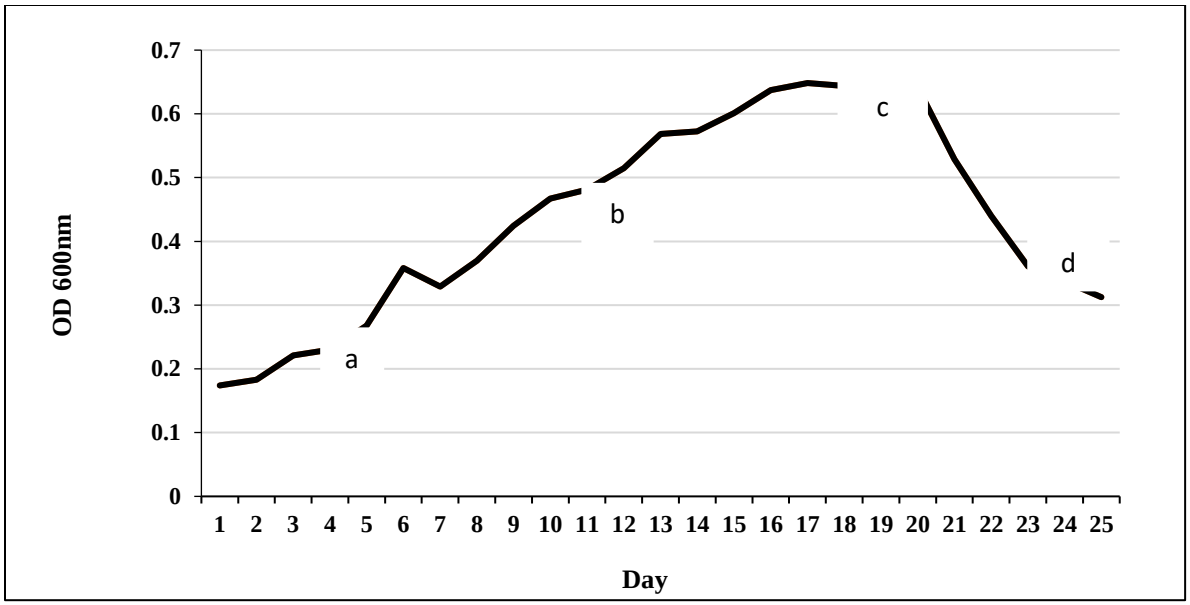


Fig 2. Growth curve of Jayapura isolate *Scenedesmus* sp., light and dark cycle of 12 hours.

Lag phase of *Scenedesmus* sp. growth was observed until day 8th. During the lag phase, cells size significantly increased and physiologically more active. In this phase, microalgae adapted to their new environment, protein synthesis and metabolism occurred but cells division has not been happened yet therefore cells density has not increased as well. Log phase of *Scenedesmus* sp. growth was observed during day 9th to day 16th. Cells division and growth rate had significantly increased to the optimum conditions. Stationary phase was occurred during day 17th to day 19th followed by dead phase.

Biomass harvested at the end of log phase was dried at 60^{o}C to constant weight in vials. As much as 0.2579 g of dried lipis was used in lipid extraction by 4 mL of n-hexane under ultrasonic frequency of 60 KHz at temperature of 60^{o}C for 120 min. Lipid obtained was 0.0784 g or 30% w/w of dried microalgae. Meanwhile, for biodiesel production, lipid in 70 mg of dried microalgal biomass was directly transesterified by adding a mixture of chloroform (0.575mL):methanol (0.425mL) (volumetric ratio of 1.35) in the presence of 50 µL of sulfuric acid (98%) and mixed 5 seconds using vortex then subsequently sonicated at 60^{o}C for 120 min. Afterward, 1 mL of deionized (DI) water was added to the mixture then centrifuged at 4500 rpm for 15 min. The organic phase was then separated well using micro pipette, dried overnight in 60^{o}C oven. Then, the produced microalgal biodiesel was analyzed for its fatty acid composition and percent of FAME using AOAC (2012):969.33 method of gas chromatography. **Fig** 3 shows the products after sonication assisted-direct transesterification and after separation of microalgal biomass.

Fig 3. Left: Microalgal biomass. Right: product after sonication and after separation

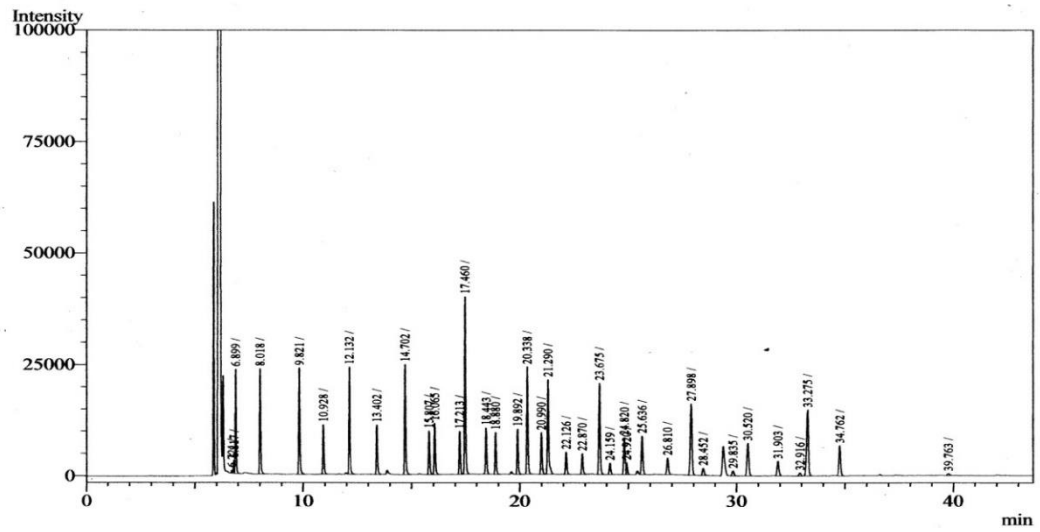


Fig 4. Spectrum of standard fatty acids

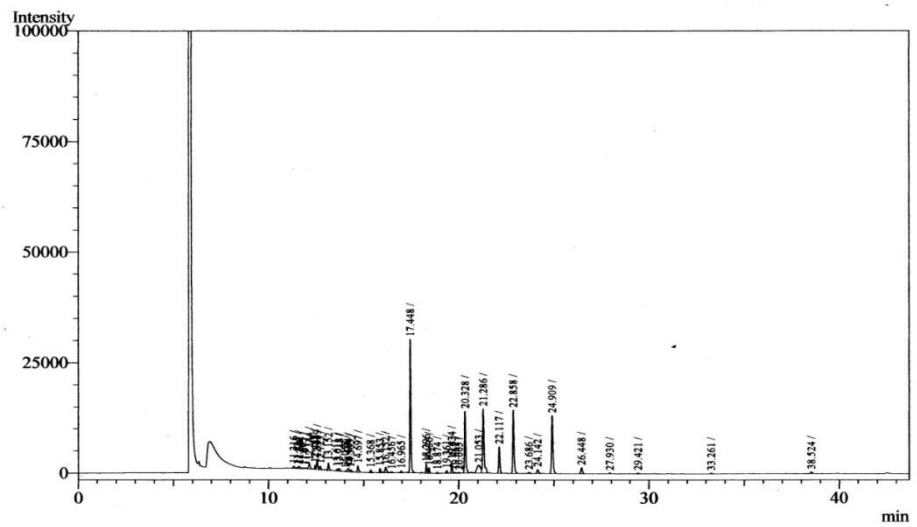


Fig 5. Spectrum of fatty acids of biodiesel produced from isolated *Scenedesmus* sp. Mikroalga Isolat Perairan Jayapura

Fig 4 and Fig 5 show the GC spectrum of fatty acids compositions of standard and produced biodiesel, respectively. The biodiesel produced has showed composition of palmitic acid (24.21%), linolenic acid (17.09%), linoleic acid (16.52%), stearic acid (12.82%), and elaidic acid (5.13%), respectively.

Conclusions

The results of this research exhibit great potential of Jayapura isolate *Scenedesmus sp*., in the production of microalgal biodiesel.

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Texture, Colour and The Preference Level for Cooked- Cured Duck Meat with Added Sodium Tripolyphosphate

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Abstract

Duck meat obtained from unproductive laying duck and old duck has a tough characteristics and dark color. Moreover, the high fat content cause the meat oxidixed easily. The previous study showed that duck meat curing in the curcumin extract could lower the fat oxidation, and the addition of the sodium tripoliphosphate (STTP) could reduce the toughness of the meat. However, meat heating during cooking could decrease the curcumin content and leach the STTP into the water. The objectives of this research was to determined the effect of cooking on the colour, texture and acceptability of the cooked-cured duck meat. The study used Randomized Complete Design, with two factors of: curcumin extract concentrations of 0.3% and 0.4% (w/w) and STPP added with various concentrations of: 0.0; 0.10; 0.15; 0.20 and 0.25% (w/w). The cooked-duck meat texture was tested by using a Texture Analyzer and the colour was tested with a Colour Reader Conica Minolta. The preference level of the cooked-cured duck meat was determined by Hedonic Test. The research showed that the cooked-cured duck meat preference was higher than that of the cooked-fresh duck meat. The duck meat cured in the curcumin extract at concentrations of 0.3% with an addition of 0.10% to 0.25% STPP resulted in the most acceptable product, based on the smell preferred, texture with high deformation and lightness colour.

Key words: cured-meat, cooking, acceptability.

Introduction

Duck (*Anas platyrhynchos*) is a type of poultry farmed for meat and eggs. The quantities of duck meat on the market are still very limited as supply mostly comes from culled females (54%), but as much as 35% can come from male salvage and up to 18% from young females (Hardjosworo, 2001). Duck meat is the meat of culled non-productive female layers and older males. Rejected duck meat has a clay-like texture and a fat content reaching 1.84%, in contrast to chicken meat at only 1.05% fat (Ali et al., 2007). Unsaturated fatty acids (ALTJ) make up more than 60% of the total fatty acids, which results in the duck meat being easily oxidized, thereby degrading flavour, destroying nutrients and leading to a build-up of toxic substances. According to Baggio and Bragagnolo (2006) meat, during processing and storage, can be the subject of oxidation induced by the presence of heat, light, metal and oxygen which will produce ROS (Reactive Oxygen Species) such as aldehydes, peroxide and cholesterol oxides that can lead to degenerative diseases such as cardiovascular disease and early aging.

Attempts to inhibit oxidation of the fat in duck meat were made by Dewi and Astuti (2013) by adding 0.3% turmeric extract as a natural source of antioxidants and then curing for 10 minutes.

Storage was undertaken for 8 weeks in a freezer. Turmeric is known to contain curcumin which can inhibit lipid peroxidation (Jayaprakasha et al., 2006). The results show that extract of turmeric can inhibit an increase in the numbers of peroxides and TBARS of duck meat, and the texture of duck meat after storage becomes more tender. But curcumin is yellow in colour and has a distinctive turmeric flavour, which can affect the acceptability of the product. In addition, the research is still limited to storage only. Yet according to Sampaio et al. (2012), lipid oxidation will continue during cooking. The problem is that the texture of duck meat becomes more tender after storage in a freezer (Dewi and Astuti, 2013), but according to Fernandes et al. (2013) storing lamb and mutton at freezing temperatures (-18 °C) causes low water retention or Water Holding Capacity (WHC), so that the texture of the meat after cooking is hard. Abdel et al. (2011) state that the addition of sodium tripolyphosphate (STPP) to lamb meat which is to be frozen can inhibit the decrease in WHC compared to the control, so the texture of the cooked meat is softer and is preferred by the consumer. And according to Marsha et al. (2013) the use of STPP in turkey meat, in addition to inhibit the oxidation of fat by slowing down the penetration of heat into the material. However, curcumin and STPP could not stable during cooking of duck meat. Hence this objective study to evaluate the effects of cooking of cured- duck meat in curcumin extract, and the addition of STPP on the physical properties and preference level of cooked-duck meat.

Materials and Methods

Materials

The materials used for the study of duck meat were derived from duck breeders in the village of Argomulyo, Sedayu, Bantul, Yogyakarta. Turmeric, as a natural source of antioxidants, was purchased from a local market in the Yogyakarta area. Analysis of the base material (duck meat) included water content (AOAC, 1990). Texture of the cooked duck meat was tested using a Texture Analyzer and colour was tested using a Colour Reader Cinica Minolta. Chemicals used in all the pro qualifying analyses were obtained from Merck.

Methods

The research method consists of five steps. These are: 1) Preparation of turmeric curcumin extract by sorting tubers, then peeling and washing. Curcumin extraction using maceration method (Marsono et al., 2005). 2) Curing fresh duck meat with turmeric curcumin extract (with a variation of 0.3% and 0.4%) and variation of the addition of STPP at 0.00; 0.10; 0.15; 0.20; and 0.25%. 3) Storage of duck meat (phase 2) at freezing temperatures (-10 °C) for 8 weeks. 4) Cooking at 100 °C during 15 minutes. 6) Testing the physical properties (texture, color), and determining the organoleptic acceptability of cooked duck meat by hedonic test.

Statistical analysis

The experimental design used was completely randomized design, factorial pattern with factors such as variation of the amount of curcumin extract and STPP, to determine the differences between treatments used by the F test, then the real difference between the samples was determined by Duncan's Multiple Range Test (DMRT) (Gacula and Singh, 1984)

Result and discussion

Texture of cooked-duck meat

The results showed that there was no interaction effect from each curcumin and STPP treatment on the hardness of the cooked-cured duck meat (Table 1). As for the texture of boiled or roasted cured duck meat, it was found to be more influenced by the addition of curcumin (Table 1). The deformation shown the toughness of cooked-duck meat. The higher the STPP the higher the deformation. It mean that the moer tender than without STPP. Soeparno (2009) states that muscle difference affects the texture and tenderness of meat, while the texture of cured duck meat which has been boiled or roasted is not affected by the addition of STPP. According to Abdel et al. (2011) STPP is generally used in cured meats to retain water during cooking, so that the palatability of the meat after cooking remains high and there is no decrease in tenderness. Therefore, in this study the texture of boiled or roasted cured duck meat is not significant (Table 1).

Table 1. Texture of cooked cured duck meat (g)

Curcumin (%)	STPP (%)					Average
	0	0.1	0.15	0.2	0.25	
0.3	532.38	624.00	698.38	500.88	600.13	591.15[a]
0.4	772.63	812.67	706.13	752.17	838.33	776.38[b]
Average (ns)	652.51	718.34	702.26	626.53	719.23	

Deformation

Deformation is a change in the meat texture caused by cutting the meat to measure texture. If the cured duck meat was overcooked, the deformation was affected by the addition of STPP. Deformation of cured cooked duck meat was greatest with the addition of STPP by 0.1%. Higher additions of STPP showed no more significant deformation. This is because in meat products, STPP is generally used to maintain texture or tenderness after cooking. In the table 2. it appears that the addition of 0.1% STPP (43.05%) was significantly different from that without STPP (37.88%).

Table 2. Deformation of cooked cured duck meat (%)

Curcumin (%)	STPP (%)					Average (ns)
	0	0.1	0.15	0.2	0.25	
0.3	38.36	42.12	41.07	35.41	38,58	39.19
0.4	37.40	43.98	37.29	42.55	40.28	40.30
Average	37.88[a]	43.05[b]	39.18[ab]	38.98[ab]	39.43[ab]	

Brightness (L)

The brightness of cooked-cured duck meat was influenced not significantly by the addition of STPP, but there was significant effect from the addition of curcumin as well as its interaction (Table 3).

Table 3. Lightness of cooked cured duck meat

Curcumin (%)	STPP (%)					Average
	0	0.1	0.15	0.2	0.25	
0.3	35.17	34.47	34.74	35.42	33.05	34.57[a]
0.4	37.13	31.44	32.22	33.65	34.60	37.85[b]
Average (ns)	34.90	32.96	33.48	34.54	33.83	

The addition of 0.4% curcumin gave the same relative brightness as the addition of 0.3%. Curcumin only serves to add antioxidants, which are substances that can inhibit an oxidation reaction in materials susceptible to oxidation (Fennema, 1996). It is likely that it has no real effect on the brightness of the flesh.

Redness

The colour of cooked cured duck meat was influenced significantly by the addition of curcumin and STPP, but the interaction was not significant (Table 4). Cooked duck meat became significantly redder with a higher addition of curcumin (0.4%) compared to a 0.3% addition. This is because adding curcumin to cooked meat gives a yellower colour, and this yellowing makes cooked meat appear redder . Ali et al. (2007) stated that the colour of the duck meat has a very high redness value, but it has a low brightness value.

Table 4. Redness of cooked cured duck meat

Curcumin (%)	STPP (%)					Average
	0	0.1	0.15	0.2	0.25	
0.3	5.54	6.73	5.99	6.04	6.54	6.17[a]
0.4	7.80	7.67	7.37	6.22	7.30	7.28[b]
Average	6.67[ab]	7.20[b]	6.68[ab]	6.13[a]	6.92[ab]	

Yellowness

Ali et al. (2007) stated that the color of the duck meat has a very high redness value, but it has a low brightness value. Dewi and Astuti (2014) stated that the addition of curcumin gave an improved flesh color.

Table 5. Yellowness of cooked cured duck meat

Curcumin (%)	STPP (%)					Rerata (ns)
	0	0.1	0.15	0.2	0.25	
0.3	20.02	24.88	23.45	20.25	21.50	22.02
0.4	22.80	20.50	20.33	23.30	23.85	22.16
Rerata (ns)	21.41	22.69	21.89	21.78	22.68	

It is further mentioned that there is a relationship between the amount of curcumin and the length of time curing the duck meat on the colour. i.e. if lower levels of curcumin are used to get the desired yellow color, then curing time has to be longer. From these results, cured cooked duck

meat is not influenced by the addition of curcumin, STPP and interaction (Table 5). This is presumably because the ripening color of curcumin will be reduced, so that the meat after cooking has the same yellow color.

Sensory Test

The sensory test of cured cooked duck meat the smell was not affected by treatment with curcumin and STPP. While colour, texture and taste were on the whole affected by treatment with curcumin and STPP, the addition of curcumin made no significant difference to the smell of the cooked meat, possibly because 0.3% and 0.4% additions of curcumin after cooking had a relatively similar smell. While the most preferred colour of cooked cured meat was achieved by adding STPP at 0.2% and curcumin at 0.3% to 0.4%. Preferred texture of the cooked cured meat is achieved by addition of curcumin 0.3% and STPP at 0.1 to 0.2%, and curcumin at 0.4% with STPP at 0.2% to 0.25%.

Table 6. Sensory Test of Cooked Cured Duck Meat

		Smell (ns)	Color	Texture	Taste	Flavor
	Normal cooked duck meat	3.67	4.40^{b}	3.13^{ab}	3.33^{ab}	3.53^{ab}
Curcumin (%)	STPP (%)					
0.30	0.00	3.20	3.80^{ab}	3.80^{ab}	4.33^{bcdef}	4.20^{abc}
	0.10	3.33	3.33^{ab}	3.19^{ab}	3.13^{a}	3.47^{a}
	0.15	3.93	3.60^{ab}	2.73^{a}	3.93^{abcde}	3.73^{ab}
	0.20	3.27	3.13^{a}	2.80^{a}	3.47^{abc}	3.30^{a}
	0.25	3.67	3.44^{ab}	3.47^{ab}	3.80^{abcd}	3.93^{abc}
0.4	0.00	3.13	3.27^{a}	4.20^{ab}	4.67^{def}	4.13^{abc}
	0.10	4.20	3.13^{a}	4.27^{b}	5.00^{ef}	5.00^{c}
	0.15	4.04	3.47^{ab}	4.33^{b}	5.13^{f}	4.67^{bc}
	0.20	4.07	3.07^{a}	3.53^{ab}	4.60^{cdef}	4.40^{abc}
	0.25	4.13	3.40^{ab}	3.20^{ab}	4.60^{cdef}	4.40^{abc}

While the preferred taste of cooked cured meat is with curcumin added at 0.3% and STPP from 0.1% to 0.25%, and curcumin at 0.4% with STPP from 0.2% to 0.25%. On the whole the most preferred cooked cured duck meat is with an addition of curcumin at 0.3% with STPP from 0.1% to 0.2%, and curcumin added at 0.3% with STPP at 0.20% to 0.25%.

Conclusion

The results of this study concluded that the cooked cured duck meat acceptability was higher than just cooked duck meat. The research showed that the duck meat cured with curcumin extract at 0.3% and 0.4% concentrations and added with 0:10 to 0:20% STPP resulted in the most acceptable product, based on texture, color and water holding capacity, especially the brighter color and softer texture after cooking.

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The Occurrence of Coral Disease Found in Dumaguete City, Philippines

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Abstract

A recent survey on coral diseases was conducted using manta tow and modified point estimate randomly done at a single point in time by snorkeling along reef sites at Piapi and Bantayan, Dumaguete City. Coral disease assessment was done by underwater photography. The diseases of corals and the characteristic of coral species affected was identified using related references. These were confirmed by subject specialists at Silliman University, Dumaguete City. The findings during dry and wet seasons in 2015 showed that there were 2 diseases: White Band Disease type II (WBD II) found in *Acropora* and tumor/Growth Anomaly (GA) found in *Porites*.

Keywords: coral disease; diseased, coral species

Introduction

Currently, coral ecosystems are reported as globally threatened under a range of climate changes that promote several diseases that increase with time, in intensity and geographic distribution (Ben-Haim and Rosenberg, 2002). The underlying causes of coral decline (or disease?) are increased temperature from 3° to 5°C above normal, salinity, and sedimentation (Kleypas et al., 1999; Goreau and Hayes, 2008). Carpenter et al. (2008) reported that one-third of all corals are in the category of risk of extinction.

A disease occurs when one of three major components (the agents, the host or the environment) break down (Merill and Timmreck, 2006). Coral disease is a phenomenon that occurs when corals lose their productive ability due to some factors. Some arise from bites of fish and invertebrates; others are due to fungi, bacteria, and virus (Ben-Haim and Rosenberg, 2002; Harvell et al., 2007; Raymundo et al., 2008; Rosenberg and Loya, 2004). The first recorded coral disease outbreak was in the late 1970s from the Indo-Pacific and Red Sea. The main causal agent of many coral diseases is bacteria influenced by environmental factors. The major causative agents are *Vibrio coralliilyticus* (Ben-Haim and Rosenberg, 2002) and *Vibrio shiloi* (Ben-Haim et al., 1999) which were found in both healthy and diseased corals. There are more than twenty coral diseases that have been described (Rosenberg et al., 2007; Sheridan et al., 2013).

The interruption of symbioses between the coral animals and their photosynthetic algal or loss of photosynthetic pigments, known as bleaching, will be the initiation of coral disease. is known as

the first sign of disease; other characteristics include changes in tissue coloration or spotting, the loss of tissue confluence, and massive cellular necrosis (Kushmaro et al., 1998; Ben-Haim et al., 1999; Rosenberg and Loya, 2004; Ritchie, 2006; Sheridan et al., 2013). In addition, shifts in coral microbiota composition may trigger the appearance of signs of diseases and/or bleaching (Krediet et al., 2013).

In 1987, 1983 and 1994, many coral reefs had been recorded bleached, with little mortality in Kenya. Unexpectedly, coral cover losses were about 40-90% in the Indian Ocean and also in Pacific reefs (McClanahan et al., 2001, 2005; Knowlton et al., 2010). Importantly, there are even new syndromes of coral-disease found in four Global Environmental Fund Centers of Excellence: the Caribbean, the Philippines, Australia, and East Africa (Harvell et al., 2007; Raymundo et al., 2005). Aside from predators and over algae growth, the findings in the field also found bacteria, viruses, protozoa or fungi can be the cause of infectious diseases on the reef that had lesions or a different band of tissue loss (Loya et al., 2001; Rohwer et al., 2001, 2002, Rohwer and Kelly, 2004, Harvell et al., 2007, Rosenberg et al., 2007; Sheridan et al., 2013).

A previous study by Raymundo et al., (2005) reported that there were five diseases and syndromes that had been identified in two regions of Philippines: the Central Visayas and the Lingayen Gulf recorded that *Porites ulcerative* appeared to have White Spot disease, tumors and pigmentation response that occurred frequently in both regions. White syndrome, described as an irregular exposed skeleton or recently *white* death marched along living tissue, may be necrotic with mucus secretion (Raymundo et al., 2008; Rosenberg et al., 2007). Brown band is caused by a ciliate that consumes the tissue of the corals found on the branching of *Acropora sp.* in Central Visayas. Growth Anomalis (GA) or tumors, as hyperplasias or neoplasias is characterized by unusual skeleton deposition, and may be accompanied by different pigmentation, disorganized corallites and few zooxanthallea (Raymundo et al., 2006; Lobban et al., 2011). Kaczmarsky (2006) identified two syndromes, *Porites ulcerative* white spot (PUWS) in massive and branching corals, coral tumor in massive corals that occurred at high prevalence while Black band disease (BBD) and had a relatively high prevalence in the islands of Negros, Cebu, Siquijor, Panglao, Olango, Sumilon, Bantayan, Pescador, Balicasag and Palawan. The aim of this study was to determine occurrence of corals diseased in Dumaguete City, Philippines.

Materials and Methods

The sampling site was in Dumaguete City (Fig 1 B and C), Philippines (Fig 1 A). The site was chosen because of the following reasons 1) presence of creeks emptying into the ocean and 2) high coral population. The actual survey was carried out in two areas: Bantayan Beach and Piapi Beach, Dumaguete City, during the dry and wet seasons in 2015.

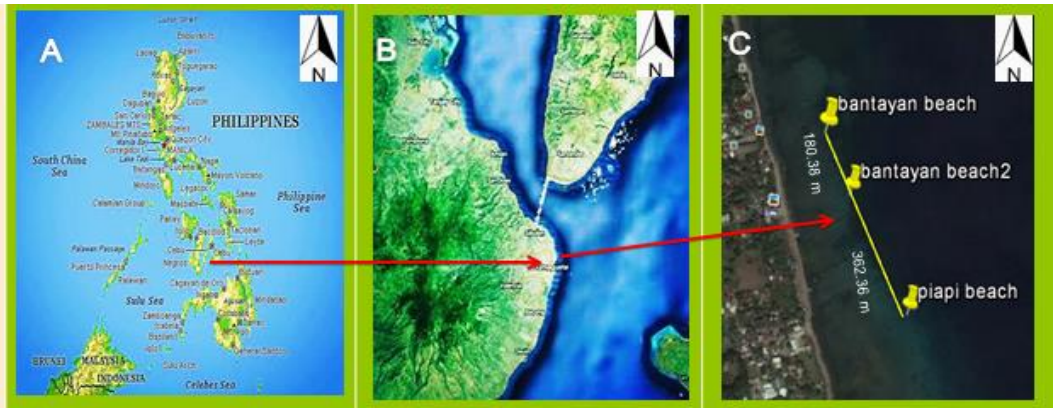


Fig 1. Map of Sampling Site (Google earth) A: Map of the Philippines; B: Map of the Dumaguete City, Philippine; C: Map showing the location of off-shore Bantayan

The first survey was made during the dry season in March and the second survey was made during the wet season in August and September 2015. The method in this study used a combination of the manta tow survey method by snorkelling hanging on outrigger bamboo on the side of an indigenous outrigger *banca* boat along reef sites excluding the MPA area and random point viewing on the coral colonies (Hill and Wilkinson, 2004; Raymundo et al., 2008). Coral disease assessments at each spot for 2 hours manta tow to identify the coral and documented in picture along the reef sites. The picture of corals species and diseases were taken with underwater photography (using Sonny underwater camera). The photos were used for further identification and confirmation. Corals were identified to genus level using the identification guides of Wallace (1999), Veron (2000) and confirmed by Clarissa T. Reboton of the Institute of Environmental and Marine Sciences (IEMs), Silliman University. Coral disease, bleaching, predation and other signs of compromised health (pigmentation response, sediment damage, algae and sponge overgrowth) were identified by the characteristics of lesions using the guides of Harvell et al., (2007), Raymundo et al., (2008) and confirmed by Kathryn B. Rosell – Jadloc of the Biology Department, Silliman University.

Results

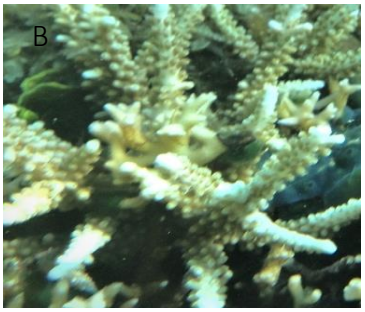
During the wet and dry seasons in 2015, werecorded two coral diseases and one syndrome at Bantayan and Piapi Beach as shown in the following data: White Band disease (WBD II) found in *Acropora*, and Tumor/Growth Anomaly (GA) found in *Porites*, (Table 1, Fig. 2, and 3).

Table 1. The occurrence of diseases in coral species in Dumaguete City, Philippines, during dry and wet seasons in 2015

Disease	**Acronym**	**Coral species**			
		Bantayan		**Piapi**	
		Dry	**Wet**	**Dry**	**Wet**
White band disease	WBD II		*Acropora*		
Tumor / Growth Anomaly	Tumor/ GA	*Porites*			
Syndrome					
Tissue Necrosis /Tissue loss/ Bleaching			*Porites* *Montipora* *Platygyra* *Acropora* *Acropora*		*Acropora* *Porites* *Ouliphyllia* *Montipora* *Echinopora*

Growth Anomaly (GA) appeared as elevated in some part of massive coral (Fig. 2.2) that was only found in dry season. Bleaching /tissue loss/tissue necrosis was the mostly observed syndrome which is caused by a specific bacterial infection found especially at the tip of the branching coral or massive coral. The bleaching or tissue loss or tissue necrosis indicated predations; this may also be brought about by beach siltation, competition between algae and disease agent, which appears in branching and massive coral as white color. Also in some corals the syndrome covered all the structure from the basal to the tip (Fig. 2.2 and Fig. 2.3). Bleaching was found in the wet season. The numbers of diseases and diseased coral species are unique in every season but the number of coral species with syndrome tissue loss/bleaching was higher during wet season found in Bantayan and Piapi beach.

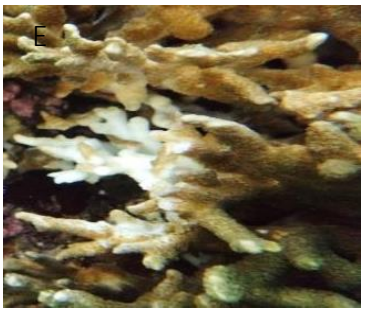
(A). *Porites*



(B). *Acropora*

(C). *Acropora*

(D). *Platygyra*



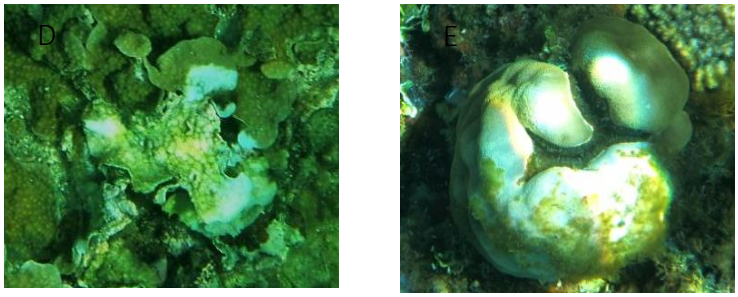
(E). *Montipora*

(F). *Acropora*

(G). *Acropora*

Fig 2. Coral diseases and syndrome profile in Bantayan and based on survey on Dry, 2015: A. Tumor/Growth Anomaly; Wet, 2015: B, C. White band diseases II (WBD II), D-G. tissue loss/bleaching (Photograph: Nusawakan (B, F, G), 2015)

(A). *Montipora* (B). *Ouliphyllia* (C). *Acropora*



(D). *Echinopora* (E). *Porites*

Fig 3. Coral syndrome profile in Piapi Beach based on survey on wet season 2015. A-E. tissue loss/bleaching (Photograph: Nusawakan (B, C, D), 2015)

The distribution of corals is not the same in every spot. There was a rich population of corals spotted in some area, while in another there was a few. Some of the corals were observed with bleaching or tissue loss. Moreover, we found that coral was covered with plastics and other garbage (in 2016 and 2017).

Discussion

Based on the surveys of diseased corals two diseases were found only in Bantayan reefs but out of the MPAs area. During dry season Tumor/Growth anomaly was found in *Porites* and in wet season was White band disease II (WBD II) was found in *Acropora* species, a clear white spot at the basal area of branching coral. While on the study of Raymundo et al., (2006) inside the MPAs area mentioned that PUWS, growth anomalies and White Syndrome were observed, and total prevalence in 7.7% for Agan-an and 7.9% for Bantayan reef. The reefs are highly impacted by untreated sewage, siltation, and agricultural runoff from this city. In addition, high population growth in the city gives an intense pressure of high fishing. The prevalence differences in these two studies are because the MPA area is considered to be well managed, has more complete communities, and may be healthier because fewer human impacts are the opposite of the areas

outside the MPA. However, MPA is often become tourist destination because of the diversity of coral and fish can promote an initiation to a disease.

The common and dominant syndrome at the sites was tissue loss or bleaching that threatened the corals at Bantayan reefs. Bleaching was found in wet season. During the wet season, the water quality was not clear or high turbidity due to some waste that was found in the ocean. Temperatures at Bantayan and Piapi reef during the data collection was 29°C in March, 26°C in August and 28°C in September 2015 which are all within the normal range (Kleypas et al., 1999). Other studies found that branching and massive corals are affected by predators that bite on the surface of the coral layers. Some of the predators were *Drupella*, *Acanthaster planci*, fish, and tube worms (Alcala et al., 1987; Hughes 1994; Raymundo et al., 2008). These were also present at Bantayan and Piapi reefs.

Conclusion

Based on the surveys of occurrence of diseased coral found in Dumaguete City during the dry and wet seasons in 2015 two diseases were identified: White Band Disease type II (WBD II) in *Acropora* species and Growth Anomaly (GA) in *Porites* species. Tissue loss/bleaching was markedly affecting the corals.

Since this survey focused on the occurrence of disease, it is important to note that when these data were gathered it was observed that the diseased part of the coral colony was minimal in comparison with the overall mass of the healthy coral.

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The Tests of Various Disinfectant and Plant Growth Regulator in MS Media Toward the Growth of *Klutuk* Banana's Explant (*Musa paradisiaca, L*)

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Abstract

This research had been conducted for 5 months from September 2017 until January 2018. This research was conducted at Tissue Culture Laboratory, Penerbangan Street, No. 87, Pekanbaru City, Riau, Sumatra, Indonesia. *Klutuk* Banana is a type of banana that is able to produce many leaves which are not easily torn and it also produces a distinctive aroma. Recently, the seedlings which are planted by the farmers are generally come from the main tree seedling so that the numbers of seedlings are limited and vulnerable to bacterial diseases and Fusarium wilt. The technique of alternative tissue culture freed the seedlings from the disease, and able to produce high quality banana seedlings in great amounts and homogeneous in a short period of time. This study used a complete randomized design (CRD), factorial with 2 factors as follows: The first factor was disinfectant which consist of levels: Sodium Hypochlorite 20% (P1), betel nut extract 20% (P2), betel (*Piper betle* Linn) leaf extract 20% (P3), Potato Arrowroot extract 20% (P4) each of which was soaked for 30 minutes by using a shaker 25-500 rpm. The second factor was various growth regulator substances on Murashige and Skoog (MS) medium which consisted of levels: 1.0 mgL^{-1} BAP (B1), 10 mlL^{-1} Bee Honey (B2), 10 mlL^{-1} Coconut water (B3), 10 mlL^{-1} bean sprout extract (B4). Each of which was conducted three repetitions and every experimental unit consisted of two bottles, and each bottle was planted one explant so there were 192 experimental units. Data observation was analyzed statistically of R-Program Application and tested in advanced by using HSD at 5% level. The results of this study can be concluded that the Disinfectant and PGR Test in Murashige and Skoog (MS) medium on explant growth of klutuk banana in vitro produced the best treatment, namely: (1) Sodium Hypochlorite Disinfectant 20% and BAP 1.0 mgL^{-1} with percentage of contamination explants 35.24%, the number of shoots 4 pieces and Percentage of living explant 65.88% (100% Synthetic). (2) The giving of Sodium Hypochlorite Disinfectant 20% and bean sprout extract 10 mlL^{-1} with percentage of Browning explants 35.24%, number of leaf 6 strands and Percentage of living explants 65.80%, (50% organics and 50% synthetic). (3) The giving of Nut Betel Extract Disinfectant 20% and BAP (Benzyl Amino Purine) 1.0 mgL^{-1} with the percentage of Browning explant 35.24%, and the shoot length 8.67 cm, Percentage of living explant 65.80% (50% Organic and 50% Synthetic). The giving of nut betel extract tisinfectant 20% and coconut water 10 mlL^{-1} was obtained the percentage of Browning explant 35.2%, shoot length 7.50 cm, and life explant percentage 62.15%, (100% Organic).

Keywords: Disinfectant, PGR, klutuk banana

Introduction

Banana plant has a dual function, the nutrients which are contained as a source of vitamins, minerals and also carbohydrates. Banana leaf is used as a wrapper of a various Indonesian traditional foods that have a specialty, because banana leaf can produce more pleasant aroma. Another traditional function is banana root tuber water can be used as a medicine for dysentery and large intestine bleeding, while the banana stem water can be used as a medicine for urinary pain and poison neutralizer (Abdurrahman, 2011). Therefore, banana is very suitable to be developed. Seedling has an important role in the development of bananas. Recently, the seedlings that are planted by the farmers are generally from the main tree seedling, so the number of seedlings' production is limited, and it is difficult to get the seedlings that are free from bacterial diseases and Fusarium wilt. Tissue culture technique is an alternative to produce high quality banana seedlings and disease-free which are obtained in large quantities, homogenous, and the short time production. Tissue culture is a technique for isolating a part of a plant such as a growing point, an organ, a group of cells or even cell, and also for growing them in an aseptic environment so that part is able to multiply by themselves into a complete new plant exactly the same as their parent.

Tissue culture requires attention on several things, namely culture media, the techniques that are used and the planting materials used must be clean from microorganisms and pathogens. Medium contamination is large enough especially the tropics can reach 95%, while the cold areas only 5%, to overcome it, the materials used must be disinfected to maximize the successful of culturing, and to be more successful it must use the right PGR which has no negative effects toward the user and the environment. Therefore, Sodium Hypochlorite that is commonly used as a disinfectant material on explant is good, but the using of Sodium Hypochlorite in a long time is not good, because Sodium Hypochlorite contains an active ingredient in the form of mercury which is very dangerous for health, so it need an alternative substitution from the organic for example; nut betel extract (tannin), betel leaf extract (tannins), potato arrowroot extract. Tannins possess characteristic such as alcohol-soluble or water-soluble because contain OH groups, able to bind heavy metals, and have anti- termites and anti-fungi substance (Carter et al 1978).

In general, growth regulators substances that are used in in-vitro cultures have three major groups, namely auxin, cytokinin and gibberellin. Auxin has a function to stimulate the root's growth, cytokinin for the growth of shoot's bud, and gibberellin for differentiation or multiplication of cell functions. Cytokinin (BAP) is a group of plant hormones which is very important as a stimulator of growth and morphogenesis used in tissue culture. BAP (Benzyl Amino Purine) is the first generation PGR of synthetic cytokinin that plays a role in raising shoots and commonly used in in-vitro culture activities, unfortunately the price is relatively expensive, it needs a substitution in the form of organic materials that is relatively inexpensive such as bee honey, Young Coconut Water and bean sprout extract. Ajibola et al, (2012), and Sari et al, (2011) in Isda et al (2016) said that honey contains some vitamins such as niacin, ascorbic acid, pantothenic acid and riboflavin. Indriani (2014) stated that coconut water is rich of potassium up

to 17%. In addition to rich of minerals, coconut water also contains sugar from 1.7% to 2.6%, and protein 0.07% to 0.55%, there are also two natural hormones namely auxin and cytokinin as supporting for cell division of coconut embryo. Soeprapto (1992) stated that the green bean sprouts were obtained by Essential amino acids which include into tryptophan 1.35%, threonine 4.50%, phenylalanine 7.07%, methionine 0.84%, lysine 7.94%, leucine 12, 90%, isoleucine 6.95%, valine 6.25%.

The aims of this study were: (1) To test the effects of disinfectant and PGR interaction on the growth of Klutuk Banana explants. (2) To find better disinfectants and PGR in the propagation of Klutuk banana seedlings in vitro in safe and healthy for laboratory workers.

Material and Methods

This research had been conducted for 5 months from September 2017 until January 2018. The research was conducted at LEMPES Network Culture Laboratory at Penerbangan Street, Rahmat Alley no. 87 Air Dingin Village, Bukit Raya District, Pekanbaru City, Riau, Sumatra, Indonesia.

The materials used in the research are shoots of Banana Klutuk (Teratak Bulu, Kampar, Riau), Disinfectant (Sodium Hypochlorite,Betel Nut Extract, Betel Leaf, Potato Arrowroot Extract), Source of PGR (Synthetic BAP, Bee Honey, Coconut Water and bean sprout extract), aquades, alcohol, MS stock , Sunlight, gelatin & sugar, spritus, label paper, universal pH paper, and aluminum voil, rubber and plastics. While the tool used in the form; machete, scalpel, bucket, belender, kater knife, meter, analytical scales, Laminar Air Flow Cabinet (LAFC), autoclave, glass measuring & erlenmeyer, Patridis & bottle, micrometer, scapel blades & tweezers, handsprayer, culture rack, spatulla, Camera, and other stationery sets.

This research used complete randomized design (CRD) with two treatment factors as follows: The first factor was Disinfectant which consisted of levels; Sodium Hypochlorite 20% (P1), Betel Nut Extract (P2) 20%, Betel Leaf Extract 20 % (P3), Potato Arrowroot Extract 20% (P4) all of disinfectant treatment were soaked for 30 minutes by using shaker. The second factor is PGR on MS medium consisting of levels; 1 mgL^{-1} BAP (B1), 10 mlL^{-1} Bee Honey (B2), 10 ml L^{-1} Coconut Water (B3), 10 ml L^{-1} bean sprout extract (B4) provided in MS medium. Each treatment consisted of three repetitions and each experimental unit consisted of 2 bottles, each bottle was planted 1 explant and there were 96 experimental units. The parameters which were observed including; percentage of contaminated explants (%), percentage of browning (%), number of leaves, number of shoots, shoot length (cm), and living explants percentage (%).Data observation was analyzed statistically of R-Program Application and tested in advanced by using HSD at 5% level.

Results and Discussion

The result of observation showed that the test of Disinfectant and PGR on MS Media interaction and singular has different effects as shown in table 1.

Table 1. Effect of Disinfectant and PGR in MS Media to explants

Treatment	The Percentage of contaminated explants (%)	The browning percentage(%)	Number of leaves (strands)	Number of shoots	Shoot length (cm)	The living explant percentage (%)
Sodium Hypochlorite + BAP	35.24 c	45.00 c	5.00 b	4.00 a	5.67de	65.88 a
Sodium Hypochlorite + Honey	45.00 bc	38.49 d	3.00 d	1.00 c	6.67 bcd	58.92 ab
Sodium Hypochlorite + Water of coconut	54.69 b	45.00 c	3.00 d	2.00 b	7.50 abc	54.70 abc
Sodium Hypochlorite + Bean Sprout Extract	45.00 bc	35.24 d	6.00 a	1.00 c	6.00 cde	54.70 abc
Betel nut Extract + BAP	51.96 b	35.24 d	5.00 b	1.00 c	8.67 a	65.80 a
Betel nut Extract + Honey	51.46 b	45.00 c	3.00 d	1.00 c	6.33 cde	54.70abc
Betel nut Extract + Coconut Water	54.69 b	35.24 d	2.00 b	2.00 b	8.00 ab	62.15 ab
Betel nut Extract + Bean Sprout Extract	45.00 bc	54.90 b	4.00 c	1.00 c	7.50 abc	65.88 a
Betel Leaf Extract + BAP	54,.9 b	45.00 c	4.00 c	1.00 c	8.50 a	45.00 cd
Betel Leaf Extract + Honey	65.87 a	54.90 b	4.67 c	2.00 b	6.00 cde	51.47 bc
Betel Leaf Extract + Coconut Water	45.00 bc	65.87 a	3.00 d	1.00 c	4.33 fg	54.70 abc
Betel Leaf Extract + Bean Sprout Extract	54.69 b	54.90 b	3.00 d	2.00 b	2.50 h	45.00 cd
Arrowroot Extract + BAP	65.87 a	45.00 c	3.00 d	1.33 c	5.00 ef	45.00 cd
Arrowroot Extract + Honey	54.69 b	51.60 b	5.00 b	1.00 c	6.00cde	38.49 d
Arrowroot Extract + Coconut Water	45.00 bc	65.87 a	4.00 c	1.00 c	6.67 bcd	45.00 cd
Arrowroot Extract + Been Sprout Extract	51.46 b	45.00 c	3.00 d	1.00 c	4.00 gh	35.24 d
HSD PB	10.91	6.08	0.44	0.44	1.51	11.97
HSD P or B	12.99	2.22	0.16	0.16	0.55	4.37
KK	5.24%	5.24%	5.24%	5.24%	5.24%	5.24%

Numbers followed by the same letter in each row and column are not significantly different based on HSD test at significant level 5%

From Table 1, it can be seen that overall the test results had a significant effect; (a) the average percentage of contaminated explants was the lowest in P1B1 treatment at 35.24%, not significantly different from P3B4, P1B2, P1B4, P2B4, P3B2, P4B3 treatment but significantly different from other treatments, (b) the average percentage of browning was also the lowest in P1B4 & P2B1 treatment at 35.24%, not significantly different from P1B2, P2B3 treatment but significantly different from other treatments, (c) the average number of leaves in which the greatest number was found in P1B4 treatment with 6 strands of leaves but significantly different from the other treatments, (d) the average number of buds was found as the highest in P1B1 treatment that was 4.00 significantly different from other treatments, (e) the average of buds length was the highest in P2B1 treatment of 8.67 cm, not significantly different from P3B1, P2B3, P1B3, P2B4 but significantly different from other treatments, and (f) the highest average percentage of living explant was found in P1B1 & P2B4 treatments of 65.88%, not significantly different from P1B2, P1B2, P2B2, P1B3, P3B3, P3B3, P1B4 treatments, but significantly different from other treatments.

Therefore, the difference occurred because the substances contained in the disinfectant (Sodium Hypochlorite) was able to reduce the percentage of contamination (a) and Percentage of browning (b) in the form of compounds where the compound was sodium hypochlorite (NaOCl) or calcium hypochlorite (Sodium Hypochlorite) which were very effective in inhibiting the development of microorganisms. Sodium hypochlorite as a disinfectant is widely used because it has beneficial properties (Rutala and Weber, 1997) in Zulkifli and Lukmanasari (2016). Other disinfectants used also contained Tannin compounds (betel nut, arrowroot and betel leaf), where Tannin has a water-soluble or alcohol-soluble, and tannin contains much phenol which has an OH group, can bind heavy metals, and has anti- termite and fungi (Carter et al., 1978). It also caused the growth of explants to be better, resulting in the highest number of leaves, the number of shoot, shoot length and percentage of living explants. Other disinfectants in the form of organic materials containing tannin compounds (betel nut, arrowroot, and betel leaf extracts), where tannin has properties of anti-termite and anti-fungi. Carter et al (1978) stated that tannin is soluble in water or alcohol because tannin contains many phenols by having OH groups, can bind heavy metals, and has anti-termites and anti-fungi. It was also stimulated with various synthetic substances (BAP) where BAP was very effective to stimulate the shoots. Muslim (2009) et al in Thahir Z, 2010) suggested to give BAP 5 ppm in MS medium was able to induce the shoot.

George and Sherington (1984) said that the cytokinins which commonly used in in-vitro culture techniques are: a.) BAP, BAP is the most effective cytokinin to stimulate the shoots. b.) Bee Honey, so, as other organic is bee honey. Bagde et al, (2013) in Isda et al (2016) that Honey contains many minerals such as magnesium, phosphorus, calcium and potassium. Ajibola et al., 2012, and Sari et al., (2011) in Isda et al, (2016) that honey contains some vitamins such as niacin, ascorbic acid, pantothenic acid and riboflavin. c.) Young Coconut Water, Young Coconut Water contains cytokinin. Muslim (2009) et al, in Thahir Z, 2010) suggested that giving BAP 5 ppm in

MS medium is able to induce the buds. Indriani (2014) said that coconut water is full of potassium (Calium) up to 17%. Besides, coconut water contains two natural hormones namely auxin and cytokinin. d.) Then the bean sprouts extract. Soeprapto (1992) said that green bean sprouts contained sucrose, fructose, and glucose, essential amino acids contained in green bean sprout are proteins such as tryptophan 1.35%, threonine 4.50%, phenylalanine 7.07%, methionine 0.84%, lysine 7.94%, leucine 12.90%, isoleucine 6.95%, and valine 6.25%.

Conclusions and Recommendations

The results of this study can be concluded that the test of Disinfectant and PGR on MS Media influence on the growth of Klutuk banana explants in vitro with the best treatment, that are: (1) Sodium Hypochlorite 20% Disinfectant and PGR BAP 1.0 mg per liter can react the Percentage of contamination explants 35.24%, Number of shoots 4 pieces and Percentage of living explants 65.88% (100% Synthetic); (2) Sodium Hypochlorite 20%, disinfectant and PGR 10% sprouted soybean extract per liter. Percentage of browning 35.24%, Number of leaves 6 strands and Percentage of living explants 65.80%, (50% synthetic and 50% organic); (3) Disinfected Extract of betel nut 20% and PGR BAP 1.0 mg per liter% by Percentage of browning 35.24%, the shoot's length 8.67 cm and Percentage of living explants 65.80%, (50% Organic and 50% synthetic) and (4), Disinfectant of betel nut extract 20% and PGR of Young Coconut water 10 ml per liter per cent by Percentage of Browning 35.24%, the shoot's length 7.50 cm and Percentage of living explants 62.15%, (100% Organic). In addition, the writer suggests (1), Use Disinfectant and PGR 100% from organic material, in order to keep the health of laboratory workers, environmentally sustainable and maximal culturing, (2) Do further research of substance which contained in betel nut extract and PGR of Coconut water that capable to replace the function of synthetic substances used in this study.

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Addendum

Research Documentation

The Growth of Planlet Sodium Hypochlorite Treatment 20% and BAP 1 mgL^{-1}

The Growth of Planlet Treatment Sodium Hypochlorite 20% and Bean sprouts Extract 10 ml L^{-1}

The Growth of Planlet Treatment 20% Coal Extract and BAP 1 mg L^{-1}

The Growth of Planlet Treatment 20% Coal Extracts and Coconut Water 10 ml L^{-1}

Transcriptome analysis of hormone-related defense gene in bananas (*Musa acuminata*) infected by *Fusarium oxysporum* f.sp. *Cubense*

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Abstract

Banana is one of the prime tropical plants from Indonesia, but there is a problem of the industry caused by disease attack on plants from pathogen Fusarium oxysporum f. sp. Cubense. One of prevention is known the pattern of early expression on banana plants due to fusarium wilt disease to get the initial profile of the banana plant defense gene associated with phytohormone. The work phase starts with the primary design with Foc conidia culture on PDA and PDB media. Plants are infected with conidia and isolated the RNA. Preparation of cDNA was done with RNA isolation result then PCR was done with a primer that had been designed and quantified with electrophoresis. The banana plants infected with Fusarium conidia has a different phenotype with control plants, the color changes on leaves become yellowish detected 6 days after inoculation. Observation of plants shows the difference of outer appearance between plants in Foc infection and control plants after 40 days, there is dryness in plants with treatment. Electrophoresis bands ERF1, ERF2, and AXR1 has down-regulation. ETR1 and GA3 have overexpression. The height of gene expression in both treatment and control depends on the functionality of each gene.

Keywords: Banana, *Defense Gene, Fusarium oxysporum*, *Musa acuminata*, *Transcriptome*

Introduction

Banana is one type of tropical plants that can grow in various regions in Indonesia but there is a problem of production in the industry caused by *Fusarium oxysporum* f.sp *Cubense* (*Foc*). This plant disease is the most dangerous disease for banana in the world because not only cause of wilting but can also cause death in plants (Visser 2010). Several hectare of banana plantations in Asia, Australia, and America are destroyed due to attacks from this pathogen. Banana have different genome such as AA, AAA, AB, AAB, and ABB. Barangan (*Musa acuminata*) is triploid AAA whis is vulnerable to *Foc* (Li *et al*. 2013).

Fusarium wilt is one of the most dangerous plant diseases in the tropics. In 1940, the disease had damaged the production of Gros Michel banana exports in Central America and the Caribbean in large numbers. Not only type Gros Michel, *Fusarium oxysporum* f.sp. *cubense* (race 4) is also able to attack other types of bananas (Agrios 2005). The Symptoms in infected banana by *Foc* are characterized by brownish-colored necrosis of the vascular tissue and yellowish on the leaf (Ploetz 2006). Farmers usually using pesticide to protect their plant, but it is not effective to against the pathogen because it can harm the environment.

Early detected symptoms on banana plants due to *Foc* attack (Fusarium wilt) is one of the ways to prevent spreading of pathogen. The initial response of plants to a stress can be observed through the process of transcription of DNA into mRNA as a molecular mechanism occurring within the cell. Transcript of mRNA (transcriptomic) may be an indication of the activation or inhibition of a gene within a particular cell and tissue in response to stress. Differences in mRNA transcripts on infected plants compared to plants can not describe the condition of the plant. It can be utilized as a strategy to reduce the spread of disease (Morcillo *et al*. 2006).

Plants always generate responses to defense against pathogens (Nürnberger & Lipka 2005), they regulate the signaling branches regulated by phytohormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) genes related to active plant defense (Sheard *et al*. 2010). These three hormones are produced in plants along with the infection of pathogens and synergistically work with defense-related gene expression such as *ERF1* (*Ethylene response factor 1*), *ERF2* (*Ethylene response factor 2*), *ETR1* (*Ethylene receptor 1*), *GA3* (*Gibberellin 3*), and *AXR1* (*Auxin resistence 1*). The expression of hormone-related defense genes will provide information on the response of plants to disease (Morcillo *et al*. 2006).

The aim of this research was to obtain a description of plant phenotype infected by *Foc* and to know the pattern of early expression profile of banana defense defense response gene associated with fitohormon, especially jasmonic acid hormone (JA), salicylic acid (SA), and ethylene (ET). The results of this study is useful to determine the information of early symptoms of plants after *Foc* infection.

Materials and Methods

Study area

The research was conducted in February 2017 until June 2017 located in molecular laboratory and green house of Al-Azhar University Indonesia. Banana plants at 30-45 days old were taken from the Tissue Culture Laboratory Lebak Bulus already acclimatized previously adapted in green house.

Procedures

Primer Design

Primers used for PCR are designed on the basis of conservative areas of defense-related hormones such as AUX (auxin), GA (giberelin), JA (jasmonate) and ET (ethylene) in some plants contained in the Banana genome database CIRAD (www.banana-genome-hub.southgreen.fr) GENE BANK or NCBI database with BLAST program (www.ncbi.nlm.nih.gov) and multiple alignment on Clustal W Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Slameto & Sugiharto 2010). The RNA sequence is analyzed to find the primary candidate using the PRIMER3 program (http://primer3.ut.ee/). Primary and efficiency as well as the presence of primary dimer were analyzed using NETPRIMER (http://www.premierbiosoft.cpm/netprimer/). Primer that has been designed then ordered and tested its activity by performing electrophoresis.

Foc Culture Preparation

The first step is inoculate *Foc* for approximately 7 days on PDA and PDB. Conidia is filtered using filter paper and then washed with distilled water. Conidia dissolved in the aquadest and calculated to 106 conidia /ml by using haemocytometer. The next step is weighing 2 grams of *Foc* mycelium and dissolved in 200 ml aquadest. After that, conidia then infected to seeds of banana plants.

Foc infection to plant treatment

Banana plants (21 plants) planted and cleaned roots using water. A total of 18 plants were given treatment and 3 other plants were used as controls that were not given treatment. The treatment group was divided into 6 groups namely H1, H2, H3, H4, and H5, and H6. The initial treatment was performed by immersing the five groups of plants within 200 ml of Foc conidia suspension for 30 minutes, while the control plants were soaked for 30 minutes in the aquades. After soaking the plants are replanted in the soil (Li *et al*, 2013).

Observation of Banana Phenotype

After *Foc* infection on banana is observation of banana phenotype. Observation of banana phenotype done every day to see the appearance of symptoms of Fusarium wilt disease. The observation held every day for 40 days.

RNA Isolation

Total RNA isolation was taken from the leaf section using GENEzolTM. The reagent is then purified by phenol chloroform isoamite (PCI) 25: 24: 1 in order to remove secondary metabolites from the sample. A total of 50-100 mg samples were smoothed with a grinder by the aid of liquid nitrogen, then transferred to a 1.5 ml sterile tube. The next step is the addition of 1 ml of GENEzolTM Reagent, followed by centrifugation process at 5000 rpm for 10 minutes. The supernatant obtained from the centrifugation was then added 0.5-1 ml PCI, then centrifuged for 13,000 rpm within 5 minutes. The coloration of supernatant color obtained using PCI as much as 2-3 times to be clear.

The purified sample with PCI was then added 200 µl of chloroform and divortex approximately for 10 s, then centrifuged at 12,000 rpm for 10 min. The next step is to precipitate the supernatant by adding 1 volumes of cold isopropanol and incubated for 10 minutes at room temperature. The result is then centrifuged at 12,000 rpm for 5 minutes. The supernatant phase is then discarded and the pellet obtained is the total RNA isolated. The pellet is then washed with 1 ml of 70% alcohol and centrifuged for 5 minutes at a speed of 12,000 rpm. The centrifugation result was then dried at room temperature for 5-10 minutes and resuspended using 50 µl ddH2O.

cDNA Synthesis

Stages performed after total RNA resuspension with 50 µl ddH2O were quantified using MaestroNano Spectrophotometer (Maestrogen Inc, http://www.maestrogen.com). After we get the good quality of RNA it is taken as much as 5 µg for cDNA synthesis. Synthesis was performed using 1 µl primary random hexamer, 1 µl dNTPs, 5 µl buffer reverse transcriptase and added ddH2O to reach 20 µl volume then incubated at 37C for 60 min.

Semiquantitative PCR Amplification

Semi-Quantitative Amplification PCR cDNA was taken as much as 2 µl to be used as PCR reaction template with Biometra PCR thermocycler. The housekeeping genes used for amplification are the ACTIN gene. The PCR composition for amplification was 2 µl total cDNA, 12.5 µl PCR mix, 1 µl primers (forward and reverse) and H2O with 20 µl reaction volume. The PCR process was pre-PRCR at 95 ° C. for 5 minutes, denaturation at 94 ° C. for 30 seconds, the primary attachment at 57 ° C. for 30 seconds, and elongation at 72 ° C. for 1.5 minutes, with 35 cycles, and post PCR at 72 ° C, 5 min, followed by 15 ° C, 10 min later PCR results in electrophoresis to see its gene band (Suharsono et al., 2008).

Data analysis

Gene expression patterns were analyzed quantitatively using the ImageJ program (Abramoff *et al.* 2004). First the intensity analysis of Ladder DNA band 1kb from Geneaid to be a reference concentration on the experimental results. Once known, each band of electrophoresis in crop then processed with ImageJ (https://imagej.nih.gov/ij/) to know the intensity to know the concentration on each band. Concentration results then analyzed with Microsoft Excell and created in the form of concentration pattern graphs.

Results and Discussion

Plant phenotype infected by *Foc*

Observations on banana plants showed the leaves 6 days after infection with *Foc* change discoloration on the lower leaves (older leaves) or commonly referred to as symptoms of chlorosis. According to Ploetz (2006), bananas that have been infected by *Foc* will experience symptoms of leaf chlorosis to yellowish on the lowest leaves. *Foc* pathogens will spread through the roots and move throughout the plant to block the vascular system, causing the yellowish color of the lower leaves followed by other young leaves to dry and ending at the death of the plant. Based on observations on plants, symptoms of post-infectious *Foc* disease look very fast. On the 6th day (Figure 1) chlorosis has occurred surrounding the lower leaf portion to a yellowish color. Plants that have been attacked will change slowly into bright yellow before it died, the spread of this disease usually spread quickly. The extermination of Foc races 4 that has attacked banana plants and plantations is not possible because *Foc* that attack plantations will rapidly destroy entire plantations (Cooperative Research Center Plant Biosecurity 2009).

Fusarium oxysporum f.sp *cubense* (*Foc*) race 4 can attack any type of banana genome that is attacked by other Fusarium races, while other Foc races can only attack multiple genomes. Foc race 1 can attack bananas with the genomes AAA, AAAA, and AAB. Foc ras 2 can only attack AAAA and ABB bananas while *Foc* race 3 only attacks ornamental plants (Nasir *et al*. 2003). Therefore an effective way to be done by farmers is by eradication of plantations because there is no banana genome that is resistant to *Foc* attacks.

Observation of plant leaves after *Foc* infection experienced a yellowish color change on the 7th day. The yellow color changes appear to be spreading as in Figure 2, day 17 shows the

discoloration of the leaves from the yellowish to dry brown. *Foc* that infect plants will block the vascular system and inhibit the translocation of water and nutrients from the roots to all parts of the plant. This causes the plants to dry and die (Department of Agriculture and Fisheries Biosecurity Queesland 2015).

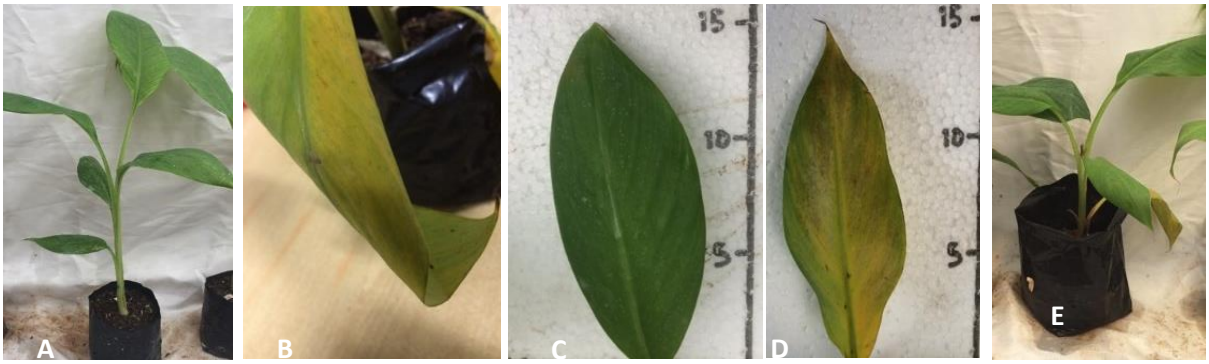

Figure 1. Phenotype of plant on the 6th day (A) Control banana plant (B) Banana plant after infection *Foc* (C) Banana leaf control (D) Banana leaf post infection *Foc* (E) Banana leaf post infection *Foc* Yellow color on leaves show symptoms chlorosis due to post-infection damage.

The process of yellowing in infected plants does not occur simultaneously on all leaves but occurs gradually starting from the oldest leaves. The yellow color widened after 7 days on the 1st leaf and spread into 2nd leaf after 9 days. The change of leaf drying faster on the first leaf after 17 days, while on the second leaf has not changed to complete dry after 17 days (Figure 2). It can occur because Foc infected the plant from the roots, so the blocking system starts wilting form the oldest root (Ploetz 2006). In contrast to plants infected by *Foc*, the first and second plant leaves on the control plants shown there is no symptoms found until the 17th day.

Figure 2. Leaves of banana plants after *Foc* infection and control on day 1,3,5,7,9,11,13 and 17. (A) The oldest leaf which is the lowest leaf on plant post infection Foc (B) 2nd leaf of the lowest part of the plant post infection *Foc* (C) The oldest leaves in the control plant (D) The second leaf from the bottom. The green leaf changes yellow and dries to brown, indicating post-infection damage.

Figure 3. Control plants and treatment plants (post *Foc* infection) on day 20 and day 40. (A) Control plants on day 20 (B) Control plants on day 40 (C) Plant by treatment on the 20th day (D) Plant by treatment on day 40.

Phenotypic observation of banana plants was done until day 40. In addition to the leaves, the differences were clearly seen in the whole plant between the treatment plants and the control plants. On the 20th day the treatment plant showed several leaves that had withered and the split rod, while the control plant had no leaves withered and the split rod (Fig. 2). This is in accordance

with a report from Monila *et al*. (2011) that the emergence of split rods is one of the plants affected by *Foc*. Plants that have been attacked by *Foc* will rapidly spread the disease throughout the plant, after experiencing the yellow color changes in all the leaves, a characteristic feature of the plant infested by *Foc* is splitting the stems then there will be drying and death on the plant as shown in plant post-infection day 40. All the colors on the leaves turn brown and dry (Figure 3D).

Semiquantitative Analysis

Semi-quantitative expression analysis of PCR with several genes such as *ERF1*, *ERF2*, *ETR1*, *AXR1*, and GA3 have different results. Differences in gene expression are represented by changes in band thickness on different days (Figure 4). *ERF1* in treatment increased expression on day 3 then again decreased on day 4 and increased on day 6. In contrast to treatment, expression on *ERF1* controls showed higher expression from day one, then increased on day 4 and decreased on day 5. *ERF2* in treatment had increased expression on day 4 and decreased expression on day 5, while expression on control increased on day 4 and decrease on day 5 as happened in *ERF2* control.

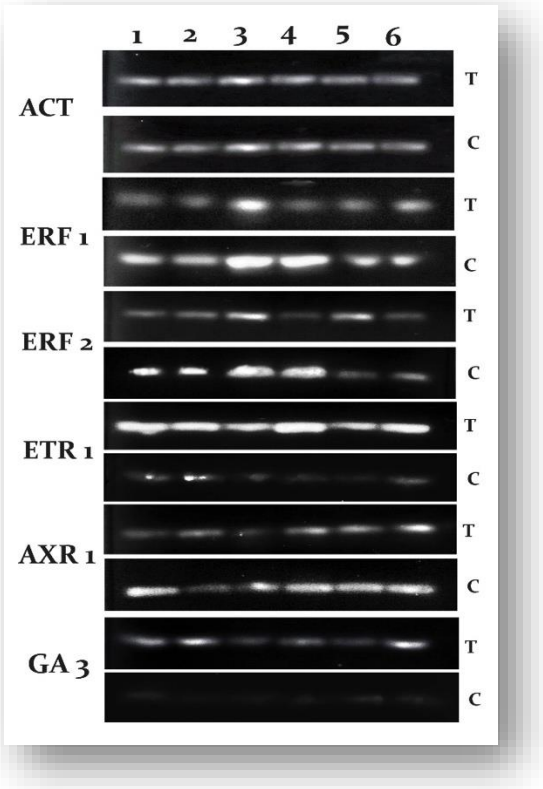

Figure 4. Semiquantitative Expression of ACT, ERF1, ERF2, ETR1, AXR1, GA3 on banana post infection *Fusarium oxysporum* f.sp *cubense*. (T) treatment (C) control (1) day 1 post infection (2) second day post infection (3) day 3 post infection (4) post-infection day 4 (5) day 5 post infection (6) 6th day post infection.

In contrast to other genes, *ETR1* experienced high expression since the first day after Foc conidia infection slightly experienced a decrease on day 5 and again increased on day 6. Controls on *ETR1* have a low expression since day 1 then increase on day 2 and decrease again on day 3. The expression of the *AXR1* gene on the treatment increased on the 4th day until the 6th day, whereas the controls showed a high expression from day one and decreased on day 2. Improved expression of *AXR1* control is again visible on Day 3 to 6th. The gene expression that occurred in *GA3* decreased on day 3 and increased again on day 6, while the controls have a constant and low expression from day 1 to day 6. The expression pattern of the ribbon is converted in graphical form in Figure 5.

Ethylene is a simple gas that is heavily involved in plant physiology. Ethylene is the most important modulator in defense against plant diseases. Generally, signal communication on ethylene has a positive role in resistance against pathogens such as Fusarium oxysporum (Van loon *et al.* 2006). Increased expression of *ERF1* in plants is a transcription that activates ethylene responsive genes that can increase resistance to pathogens. Based on research conducted by Li et al. (2013) in Arabidopsis, *ERF* gene was found with high expression after infection by *Fusarium oxysporum* f.sp *cubense* (*Foc*) after day 2. In accordance with these studies, increased expression of *ERF1* and *ERF2* in banana plants can occur due to the defense performed by post-infectious ethylene and looks high on day-to-3. The existence of the defense then allows the decrease of expression on the day after that is the 4th day.

Genes *ETR1* cooperates in signaling with other genes and hormones in response to stress, one of which is PR (Pathogenesis related). High ethylene signaling can increase resistance to pathogens. The expression of ETR in tomato plants infected by Ralstonia solanacearum was elevated followed by rapid high expression of the PR gene, but a higher and fast expression was shown in tomato plants infected by Xanthomonas campestris (Zhang et al., 2004). Expression of ETR increasing also in tobacco plants affected by *Peronospora hyoscyami* f. sp. *tabacine*, but did not arrage response to tobacco plants attacked by the Tobacco mosaic virus (Geraats *et al*, 2003). This suggests that the same genes in the same species may produce different responses, depend on the type of pathogen.

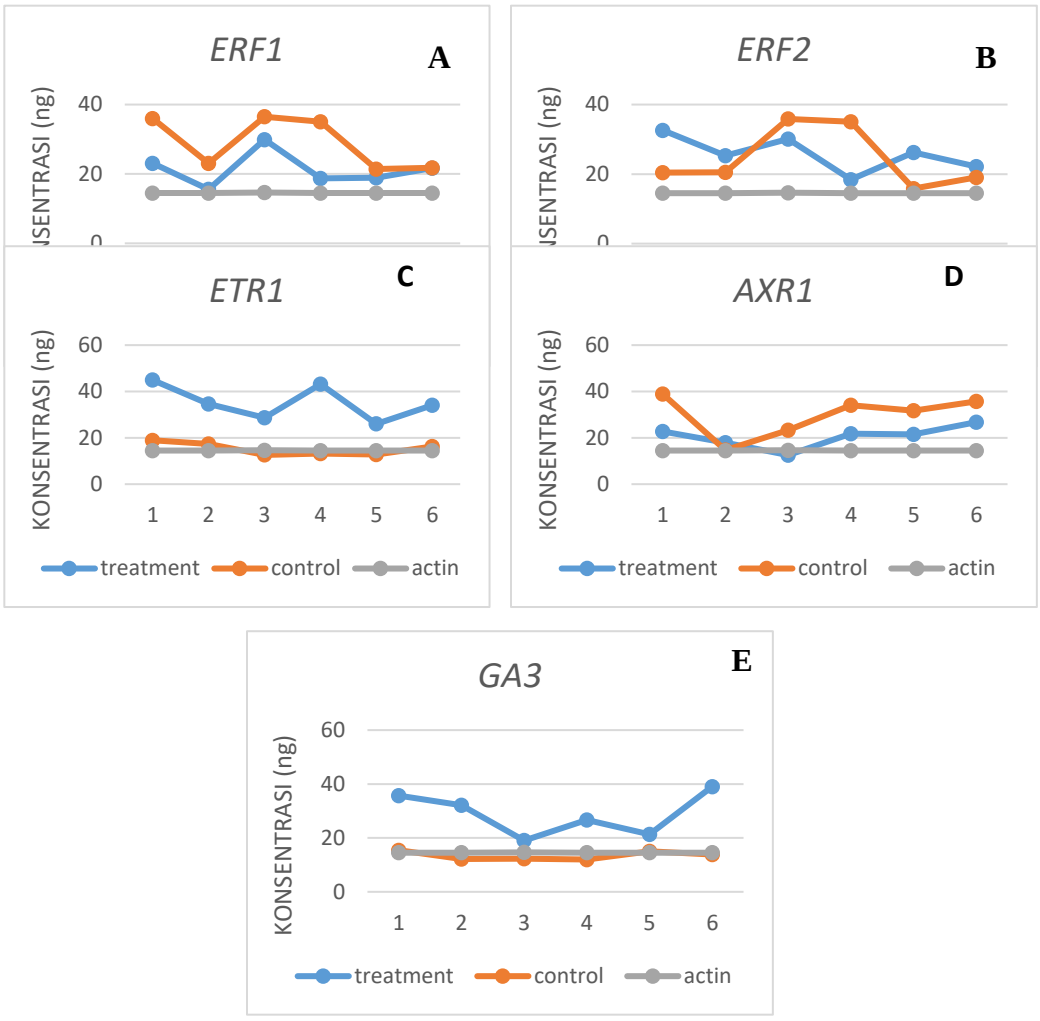

Figure 5. Graph of expression pattern on *ACT, ERF1, ERF2, ETR1, AXR1, GA3* genes. (A) Expression pattern on actin, control, and treatment on *ERF1* (B) Expression pattern on, control, and treatment on *ERF2* (C) Expression pattern on actin, control and treatment on *ETR1* (D) Pattern of expression on actin, control, and treatment on *AXR1* (E) The expression pattern on actin, control, and treatment on *GA3*. Data expression pattern is taken from the result of ribbon image analysis with ImageJ.

Conclusions

Phenotypic observations on banana plants showed symptoms of chlorosis after 6 days after infection in the form of discoloration of the leaves to yellow and change color to brown after 17 days on the lowest leaf and followed by chlorosis in younger leaves. Observation of plants up to day 40 shows the difference of outer appearance between plants in Foc infection and control plants, control plants are still green until day 40 while plants that have been infected are brown and dry. The expression of semi-quantitative real time PCR with several genes such as *ERF1*, *ERF2*, *ETR1*, *AXR1*, and *GA3* have different results. *ERF1* experienced an increase in expression on day 3, ERF2 had an increase in expression after day 4, ETR1, decreased on day 5 AXR1 experienced a 4th and

6th day, and GA3 decreased on day-to- 3. The height of gene expression in both treatment and control depends on the functionality of each gene.

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