

Effects of Nitrate and Salinity on Fatty Acid Composition of Marine *Tetraselmis sp.*: Potential as Biodiesel

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Abstract

Identifying sources of renewable energy is extremely important. The potential of *Tetraselmis sp.* in biodiesel production was investigated using a completely randomized design with four treatments. *Tetraselmis sp.* was cultured on media containing different concentrations of nitrate there are 2 mM and 15 Mm and s 25 ‰ and 35 ‰ salinity. The treatments namely are (N₂S₂₅), (N₂S₃₅), (N₁₅S₂₅) and (N₁₅S₃₅). Analysis of the fatty acid content using gas chromatography-mass spectrometry (GC-MS). The results showed that the highest growth occurred in culture that contained 15 mM of nitrate and 35 ‰ salinity. The lowest growth occurred in culture containing 2 mM of nitrate and 25‰ of salinity. The highest lipid content was found in cultures containing 2 mM of nitrate and 25‰ salinity, it is 34.83%. Seven fatty acids were detected in culture containing 2 mM of nitrate and 25‰ salinity namely myristic acid (4.02%), palmitic acid (40.59%), palmitoleic acid (29.06%), stearic acid (0.95%), oleic acid (12.52%), gamma-linolenic acid (2.56%), and arachidonic acid (9.38%). Four fatty acids palmitoleic acid (8.99%), palmitic acid (37.34%), oleic acid(44.89%), and stearic acid (8.78%) were detected in 2 mM of nitrate with 35‰ salinity. The fatty acids have potential to be used as raw material for biodiesel production.

Abstrak

Efek Nitrat dan Salinitas terhadap Mikroalgae Air Laut *Tetraselmis sp.* sebagai Bahan Biodiesel. Sumber energi terbarukan perlu diteliti. Penelitian untuk mengetahui potensi *Tetraselmis sp.* untuk biodiesel dilakukan menggunakan Rancangan Acak Lengkap pada 4 perlakuan. *Tetraselmis sp.* Mikroalgae dikultur pada medium yang mengandung nitrat dan salinitas pada beberapa formula yaitu 2 mM25‰ (N₂S₂₅), 2 Mm 35‰ (N₂S₃₅), 15 mM 25‰ (N₁₅S₂₅), serta15 mM35‰ (N₁₅S₃₅). Kandungan asam lemak dari *Tetraselmis sp.* diuji menggunakan *Gas Chromatography-Mass Spectrometry* (GC-MS). Hasil menunjukkan pertumbuhan tertinggi terjadi pada kultur N₁₅S₃₅, sedangkan pertumbuhan terendah terjadi pada kultur N₂S₂₅. Kandungan lipid tertinggi ditemukan pada *Tetraselmis sp* yang dikultur pada medium N₂S₂₅ yaitu 34.83%. Hasil penelitian terhadap asam lemak menunjukkan adanya 7 jenis asam lemak dari *Tetraselmis sp* yang dikultur pada N₂S₂₅ yaitu asam myristic (4.02%), asam palmitic (40.59%), asam palmitoleic (29.06%), asam *stearic* (0.95%), asam oleic (12.52%), asam gamma-linolenat (2.56%), dan asam arachidonic acid (9.38%). Sementara itu pada *Tetraselmis sp* yang dikultur pada kultur N₂S₃₅ terdeteksi 4 jenis asam lemak, yaitu: asam palmitoleic (8.99%), asam palmitat (37.34%), asam *oleic* (44.89%), dan asam *stearic* (8.78%). Asam-asam lemak tersebut berpotensi untuk dijadikan bahan pembuatan biodiesel.

Keywords: biodiesel, fatty acid, nitrate, salinity, Tetraselmis.

Introduction

Based on data from the Ministry of Energy and Mineral Resources (ESDM) in 2006 [1], Indonesia is dependent on several resources for energy. These include oil (46.99%), natural gas (21.8%), coal (23.5%), hydropower (2.4%), and geothermal energy (5.5%). The nation's

energy consumption is predicted to continue increasing with the growth of transportation and industry, both of which are major consumers of petroleum. All human life on earth requires energy. As oil is not renewable, we need to take to secure a domestic energy supply. Microbial biodiesel production is an alternative renewable energy to solar-based fuels. It can be derived

from vegetable oil by transesterification and esterification.

In general, biodiesel is produced from palm oil, sunflower, soybean, and canola. However requires a vast land area and may cause damage and threaten food security [2]. Different with biodiesel from marine microalgae, it doesn't need crop land area. As an alternative to plant-based biodiesels, researchers are concentrating their efforts on the production of biodiesel from marine microorganisms, which do not cause environmental damage or use valuable crop land.

Marine microalgae they have several advantages for the examples, saline or marine water can be used for culture, thereby not depleting fresh water supplies. In addition, microalgae can absorb CO₂ for photosynthesis. Depending on its productivity, certain species of microalgae may produce more than 30,000 L of oil or about 200 barrels per acre compared with 100,000 L/ha produced by soybean cultivation [3].

The biochemical composition of microalgae depends on environmental conditions and macronutrients, such as nitrogen and phosphorus, and carbon sources [4]. *Tetraselmis* sp. which cultured in medium supplemented with phosphorus (20 mL/L) showed growth increasing, but the lipid yield was lowest ($9.41 \pm 0.27\%$ dry weight). Higher lipid yields were observed at lower phosphorus concentrations. The study also showed that in CO₂ supplementation (10%) and nitrogen uptake (0.316 ± 0.08 NH- N /cell/day indicated increasing of lipid content ($10.95 \pm 15\%$ dry weight) [5].

The present study focused on the sea microalgae *Tetraselmis* sp. which has the potential to accumulate almost 50% of its dry weight as lipids [6]. Based on a previous study, modifying the composition of growth media can increase microbial growth and lipid levels [7]. The aim of the present study was to enhance the growth and lipid levels of *Tetraselmis* sp. by culturing the microalga in media containing different concentrations of nitrate and different levels of salinity.

Materials and Methods

This research was divided into three phases. In the first phase, *Tetraselmis* sp. from Balai Besar Pengembangan Budidaya Laut, Lampung was grown in growth medium containing 1 L of marine water collected from Sea World Indonesia in Jakarta and Conwy/Walne medium consisting of 1 mL of macronutrients, 1 mL of micronutrients, and 0.1 mL of vitamins. The laboratory equipment and marine water were sterilized by digital autoclaving at 121 °C, 2 atm for 15 min. *Tetraselmis* sp. was cultured with a stone aerator in 100 mL, 250 mL, 500 mL, 1 L, 2 L, and 5 L of growth medium. An 18-watt fluorescent lamp was used as a source of light. *Makara J. Sci.*

Comparison of *Tetraselmis* sp. against growth medium was 30% (v/v).

In the second phase, the microalga was cultured in several concentrations of nitrate and salinity in 10 L of growth medium. Concentration of nitrate were 2 mM and 15 mM, and salinity were 25‰ and 35‰. In the third phase, the microalgae was exposed to four different treatments: medium consisting of 2 mM of nitrate and 25‰ salinity, 2 mM of nitrate and 35‰ salinity, 15 mM of nitrate and 25‰ salinity, and 15 mM of nitrate and 35‰ salinity. The growth, lipid concentration, and fatty acid lipid composition were then analyzed.

The research used a completely randomized design, and ANOVA test was used to analyze the data. If the factors of treatment were significantly different according to the ANOVA test, Least Significance Difference (LSD) test was conducted. A value of $\alpha = 5\%$ was considered a significant difference between the standard treatment and its interaction.

Culture of *Tetraselmis* sp. Before culturing in different saline and nitrate concentration *Tetraselmis* sp. were derived from pure isolates. It increased by using 100 mL, 250 mL, 500 mL, 1 L, 2 L, up to 10 L of growth medium. The cell density was increased for six to seven days. After the seventh day of culture, it was moved to larger media. To maintain a supply of pure isolates, its liquid form was kept in refrigerator. Thus, its frequent agitation was preserved to prevent precipitation, and it was useful for air circulation.

After the amount of *Tetraselmis* sp. cells were enough for a treatment, the cultures will be taken with salinity and nitrate treatments. The aquarium was filled with seawater which has been assorted by Conwy/Walne and nitrate in a volume of $28 \times 22 \times 17.5$ cm³ a capacity of 10 L. The inoculation may reserve microalgae for 30% (v/v) due to the used medium. Then, the culture, which was ready to be paired on airstone and hose, was connected to the aerator. The culture was placed in a culture chest, with an 18-watt fluorescent lamp. Meanwhile, the culture was used to calculate the growth curve to determine the period of time in undertaking subcultures and gaining microalgae cells to compute lipid extraction and its dry weight. The population of microalgae was calculated with a haemocytometer under a microscope.

The biomass of microalgae was produced by filtering the cells using microfilter paper. The accumulated biomass was transferred to a wide petri dish and dried with fluorescent light radiation.

Lipid extraction with the Soxhlet method. Samples dry biomass 1-3 g were wrapped in filter paper. Then, The filter paper was placed in the Soxhlet. The

condenser was mounted upon the top and underneath the fat. Hexane was placed in a flask with a volume of fat Soxhlet at one quarter. The minimum period of time to use reflux was 5 h due to the solvent was clear. The hexane solvent in the flask was distilled to obtain lipids from fat pumpkin. The fat pumpkin containing the lipid extract was heated in an oven at 80 °C for 1 h to remove residual water and any remaining solvent. Once it was dried to keep its cool and weight in a desiccator, lipids were also be pondered. The weight of lipids was calculated as follows:

$$\% \text{ lipid} = \frac{\text{lipid weight (g)}}{\text{sample weight (g)}} \times 100\% \quad (1)$$

Analysis of fatty acids with gas chromatography-mass spectrometry (GC-MS). The extracted lipid samples were analyzed with GC-MS to determine the components of fatty acid. Before it was injected into the GC-MS, a sample of transesterification reaction was conducted with 4.5 mL of 0.5N NaOH in methanol, vortex, the heating of water bath within 5 min, and kept cool. Then, a 14% solution of BF₃ was added to 3 mL of methanol, vortexed, heated, and cooled. The addition of n-hexane (grade chromatography) in samples, vortex, heat it and keep it cool to perform the centrifugation at 3000 rpm. The samples (5 µL) were transferred into GC tubes ready for injection into the GC column. The samples were injected into a 30 m column of dimethyl polysiloxane with a thickness of 0.25 µm df at a temperature of 80 °C. The GC was ran with the hexane solvent. The injector temperature was 250 °C, and types of split injector. The starting temperature was 80 °C. This was maintained for 2 min and increased to 210 °C at 10 °C per min at intervals of 1 min. The temperature was increased again to 280 °C, with an increase of 6 °C per min and held for 10 min. The last injection phase was at a temperature of 280 °C.

Results and Discussion

Indicating of *Tetraselmis* sp. growth is increasing of cell density and the green color of the culture became stronger. The exponential phase occurred from the third to the fifth days. The stationary or death phase started on the third or fifth day.

Growth of *Tetraselmis* sp. The culture medium affects the growth of microalgae [8]. Figure 1 shows the growth curve of *Tetraselmis* sp. in the lag phase, exponential phase (logarithmic), stationary phase, and death phase. The lag phase of *Tetraselmis* sp. had a short duration of two days. This may be related to the initial inoculation (v/v) of the cell growth media with 30% microalgae. The length of the lag phase depends on the growth media and environmental conditions, as well as the age and number of inoculums. The metabolism when lag phase which occurred in the cells *Tetraselmis* sp. but

was not involved the activity of cell division so that the cell density was not increased. The cell density of *Tetraselmis* sp. increased in the exponential phase (logarithmic) due the growth of the cells. In this phase, photosynthesis produces carbohydrate, and protein synthesis provides proteins for growth and increased cell division [9]. In the stationary phase, the limiting factor and the growth rate were balanced, which resulted in a relatively constant cell density [10]. The interaction between the effects of nitrate and the influence of salinity significantly affected the number of cells (Fig 1). According to the ANOVA test, the interactive effects of nitrate and salinity affected cell growth from the third to the ninth day but not on the sixth and seventh day. On the third day, the highest and lowest numbers of cells were found in the N₁₅S₂₅ (1.51×10^6 cells/mL \pm 0.02 \times 10⁶) and N₂S₂₅ treatments (1.07×10^6 cells/mL \pm 0.02 \times 10⁶). However, on the fifth day, the highest and lowest number of cells were found in the N₁₅S₂₅ and N₂S₂₅ treatments (1.95×10^6 cells/mL \pm 0.03 \times 10⁶ and 0.66×10^6 cells/mL \pm 0.01 \times 10⁶). At the end of the experiment on the ninth day, the highest numbers of cells were observed in N₁₅S₃₅ (0.92×10^6 cells/mL \pm 0.03 \times 10⁶) treatment and the lowest numbers in the N₂S₂₅ treatment (0.31×10^6 cells/mL \pm 0.02 \times 10⁶),

Nitrate and salinity influence the growth *Tetraselmis* sp., with previous research reporting that a low level of nitrogen in macronutrients may limit the growth of cells [8]. Another study reported that a high concentration of carbon and nitrogen affected the growth of *Chlorella* sp. 227 [11].

Therefore, the stationary phase may easily to achieve within its cultures with low nitrogen. A low level of nitrate may also have an adverse effect on the metabolism of *Tetraselmis* sp., cell division and thereby reducing cell population levels. Previous research demonstrated that low levels of nitrogen in media reduce the productivity of microalgae [12]. Nitrate is a source of nitrogen in carbon

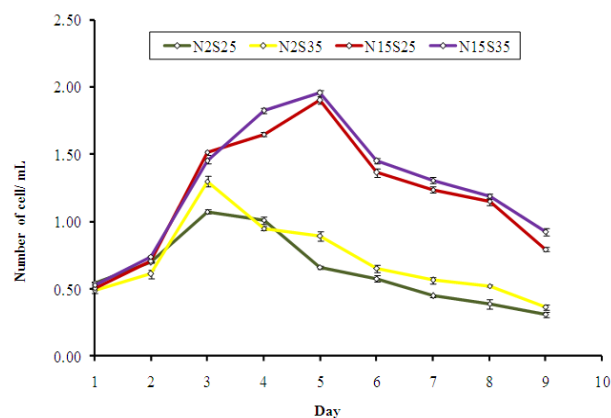


Figure 1. Curve of *Tetraselmis* sp. Culture Growth with Nitrate Concentrations and Levels of Salinity

carbon synthesis of *Tetraselmis* sp. The process of photosynthesis involves the chlorophyll pigment of *Tetraselmis* sp. As shown in an earlier study, the amount of chlorophyll decreases in accordance with a reduction in the concentration of nitrogen in culture media [13]. The reduction in chlorophyll also hampers the processes of photosynthesis and assimilation, which, in turn, inhibits growth.

Salinity affects the growth and the productivity of microalgae, with research showing the growth rate of microalgae varies according to the level of salinity in the surrounding environment [9]. As demonstrated previously, *Tetraselmis* sp. from Penang National Park coastal water can be grown under 16 g/L salinity [14]. In the present study, *Tetraselmis* sp. grew at salinity levels of 25‰ and 35‰. Based on research that the optimum growth of *Tetraselmis* sp. may apply to 20–35 ppm salinity [15].

Dry weight (biomass). The dry weight was determined at the end of the growth phase and on the ninth day (death phase) in 10 L medium culture. The biomass of *Tetraselmis* sp. was determined by estimating the dry weight of $4.02 \text{ mg}/10^6$ cells [16].

On the third day at the end of the exponential phase, the highest biomass dry weight in the $N_{15}S_{25}$ treatment in 10 L was 6.07 g. In the N_2S_{25} treatment, it was 4.30g. On the fifth day, the highest biomass dry weight was 7.83g in the $N_{15}S_{35}$ treatment. The lowest in the N_2S_{25} medium was 2.65 g. In the death phase (ninth day), the highest biomass dry weight in the $N_{15}S_{35}$ treatment was 3.69 g, and the lowest biomass dry weight in the N_2S_{25} treatment was 1.24 g. On the ninth day, the highest dry weight of *Tetraselmis* sp. was 3.68 g in the $N_{15}S_{35}$. The data shows that increasing the nitrate concentration to 15 mM and the salinity 25-35‰ can increase the biomass dry weight of *Tetraselmis* sp.

Level of lipids. Lipid levels were measured at the end of each growth phase, the end of the exponential phase (third and fifth days), and the end of the final death phase (ninth day). The results showed that the lipid levels were highest (11.83-34.83%) at the end of the death phase and lowest (4.22-26.89%) at the end of the exponential phase. The ANOVA test results revealed significant effects ($p < 0.01$) of the concentration of nitrate and salinity levels on the content of *Tetraselmis* sp. Based on the results of the LSD, every combination of nitrate and salinity treatments were significantly differ from each other (Figure 2). The highest and lowest lipid levels were found on the third day in the N_2S_{25} and $N_{15}S_{35}$ treatments ($26.89\% \pm 0.38\%$ and $4.22\% \pm 1.02\%$, respectively). The highest and lowest levels of lipids were observed in the N_2S_{25} and $N_{15}S_{35}$ treatments ($32.5\% \pm 0.50\%$ and $7.83\% \pm 0.76\%$, respectively) on the on the fifth day. On the ninth day at the start of the

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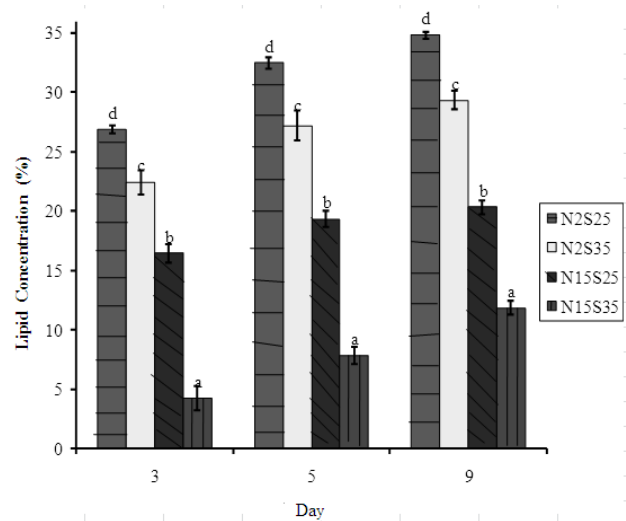


Figure 2. The Effect of Nitrate and Salinity on Lipid Levels (%) of *Tetraselmis* sp. in the Late Exponential Phase (Third and Fifth Days) and the Death Phase (Ninth Day). The Mean Values are \pm SD, $n = 3$. The Different Letters on the Bar Graph Indicate a Different Value based on LSD $p < 0.05$

death phase, the highest and lowest levels of lipids were produced in the N_2S_{25} and $N_{15}S_{35}$ treatments ($34.83\% \pm 0.29\%$ and $11.83\% \pm 0.58\%$, respectively).

The productivity of *Tetraselmis* sp. is affected by environmental factors and the composition of the culture media. Based on this study, nitrate increased the amount of biomass but not the yield lipid. Lipids are synthesized by the formation of gliserolaldehyde in the process of glycolysis and Co-A acetyl in the decarboxylation of pyruvic acid. Glycerol and fatty acids are converted to lipids [3]. The high level of lipids in the N_2S_{25} treatment in the stationary phase may be caused by the process of synthesizing, which the biomass in logarithmic phase was quick due to the limited availability of nitrogen. In addition, the metabolism of the cells changed, switching from the formation of cell structure to lipid synthesis.

The biochemical composition in the exponential phase is different from that in the stationary phase [17]. In this study, the types of fatty acids produced in the N_2S_{25} and N_2S_{35} cultures differed because of the effect of the different nitrate and salinity treatments. As shown in a previous study, the content of fatty acid in microalgae may vary based on its growth conditions [18].

Test of gas chromatography (GC). The two N_2S_{25} and N_2S_{35} treatments with the highest lipid levels on the ninth day were analyzed with GC. Seven types of fatty acids (myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, gamma-linolenic acid, and arachidonic acid) were detected in the N_2S_{25} treatment (Table 1). The treatment of chromatogram on N_2S_{35}

Table 1. Fatty Acids with N₂S₂₅ Treatment

Fatty acids	Lipid number	Area (%)
Myristic acid	C _{14:0}	4.02
Palmitic acid	C _{16:0}	40.59
Palmitoleic acid	C _{16:1}	29.06
Stearic acid	C _{18:0}	0.95
Oleic acid	C _{18:1}	12.52
Gamma-linolenat acid	C _{18:3}	2.56
Arachidonic acid	C _{20:4}	9.38

Table 2. Fatty Acids with N₂S₃₅ Treatment

Fatty acids	Lipid number	Area (%)
Palmitoleic acid	C _{16:1}	8.99
Palmitic acid	C _{16:0}	37.34
Oleic acid	C _{18:1}	44.89
Stearic acid	C _{18:0}	8.78

Table 3. Comparison of Fatty Acids (%) Produced by Some Raw Biodiesel Materials

Raw biodiesel materials	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenat acid	Other fatty acids
	(C _{16:0})	(C _{16:1})	(C _{18:0})	(C _{18:1})	(C _{18:2})	(C _{18:3})	
Oil palm*	42.6	0.3	4.4	40.5	10.1	0.2	1.1
Soybean*	11.9	0.3	4.1	23.2	54.2	6.3	-
Sunflowers*	6.4	0.1	2.9	17.7	72.9	-	-
Corn*	6.0	-	2.0	44.0	48.0	-	-
<i>Tetraselmis</i> sp. **	15.33	3.23	0.43	12.25	9.66	16.17	-
<i>Tetraselmis</i> sp. (N ₂ S ₂₅)***	40.59	29.06	0.95	12.52	-	2.56	14.31
<i>Tetraselmis</i> sp. (N ₂ S ₃₅)***	37.34	8.99	8.78	44.89	-	-	-

References: * (19), ** (21), *** This research

detects fatty acid, there were palmitoleic acid, palmitic acid, oleic acid, and stearic acid (Table 2). The highest concentrations of fatty acids were found in the N₂S₂₅ treatment. Palmitic acid (C_{16:0}) and palmitoleic acid (C_{16:1}) of 40.59% and 29.06%, respectively. Palmitic acid (C_{16:0}) and palmitoleic acid (C_{16:1}) was produced in the N₂S₂₅ treatment more than in the N₂S₃₅ treatment.

The type of fatty acid in N₂S₃₅ treatment is oleic acid (C_{18:1}) and palmitic acid (C_{16:0}) respectively 44.89% and 37.34%. Both of fatty acids have potential as biodiesel.

As reported previously, the use of saturated fatty acids in biodiesel production results in better oxidation and fewer emissions than unsaturated fatty acids [19]. Research has also shown that palmitoleic acid (C_{16:1}) has a greater advantage as biodiesel than oleic acid [20] because high levels of oleic acid increase the resistance to oxidation [18].

The highest level of oleic acid (44.89%) was produced by *Tetraselmis* sp. in the N₂S₃₅ treatment. The level of acids produced was better than that found with other sources of crude oils, such as oil palm, soybeans, sunflowers, and corn (Table 3). The palmitic acid content was 37.34% and in N₂S₂₅ was 40.59%. Linoleic acid was the dominant fatty acid component (23.06%) when *Tetraselmis* sp. was cultured in the marine water and Conway medium (4:1). Based on these data, the type of fatty acids produced by *Tetraselmis* sp. is dependent on the composition of the culture medium [21].

The best optimum treatment to increase the growth of *Tetraselmis* sp. was a concentration of 15 mM nitrate
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and a salinity level of 35%. The treatment also showed the highest biomass dry weight was 7.83 g. The best optimal treatment to increase lipid levels of *Tetraselmis* sp. was a concentration of 2 mM nitrate and a salinity level of 25% ($p < 0.05$), it is 34.83%. Seven types of fatty acids there are myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, gamma-linolenic acid, and arachidonic acid were detected in the treatment with 2 mM nitrate and salinity of 25%. Four types of fatty acids (palmitoleic acid, palmitic acid, oleic acid, and stearic acid) were detected in the 2 mM nitrate and 35% salinity treatment. The different concentrations of nitrate and salinity levels exerted a significant effect ($p < 0.05$) on the growth of *Tetraselmis* sp. and the kinds of fatty acids produced.

Acknowledgements

Thank you to Direktorat Penelitian dan Pengabdian kepada Masyarakat Kementerian Pendidikan Nasional (DP2M) Republik Indonesia for research funding in 2009. We also thank for Lembaga Penelitian dan Pengabdian Masyarakat (LP2M) UAI and Dr. Ir. Witono Basuki, M.Sc., dean of Mathematics and Natural Science Faculty UAI in 2008 who has given the laboratory facilities.

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