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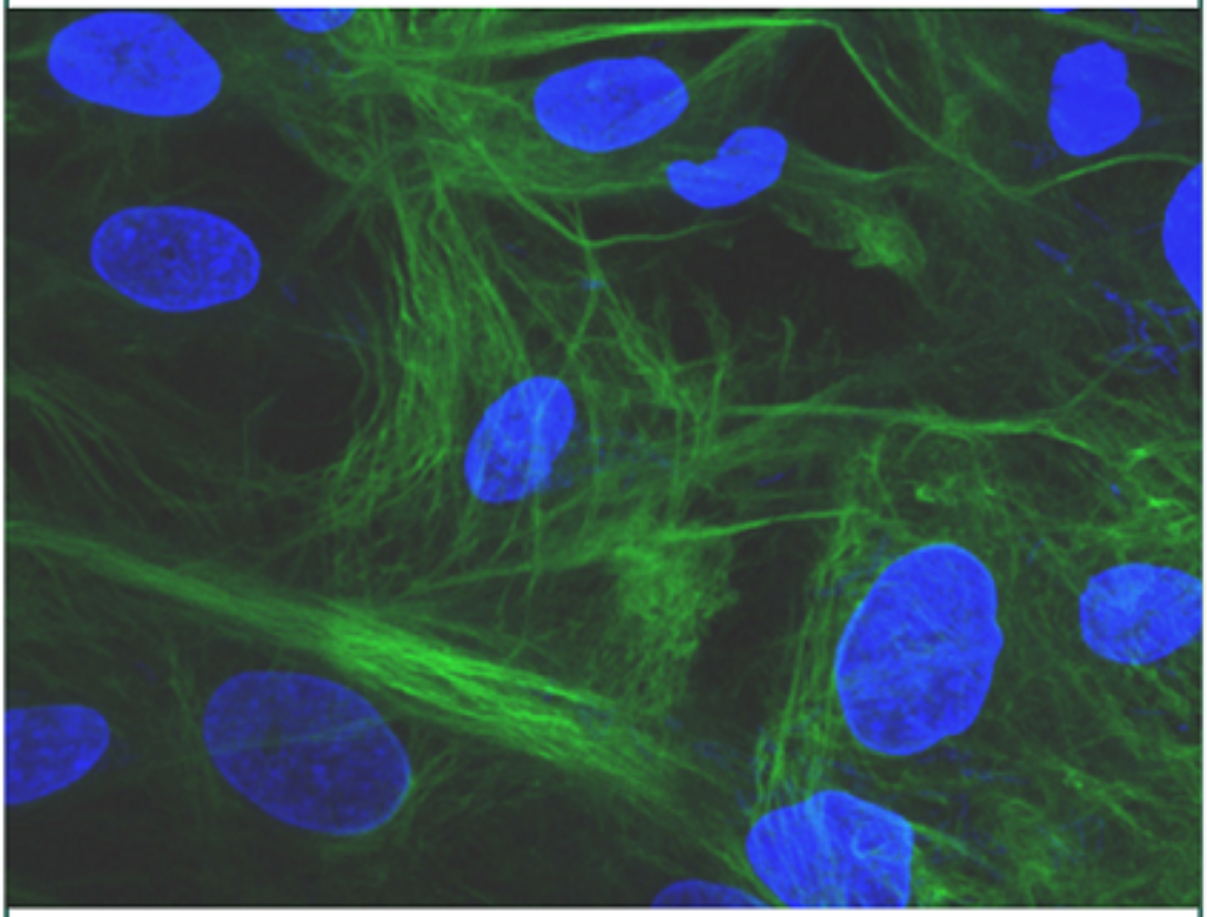

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School of Life Sciences and Technology
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The Protective Effect of Propolis Nanoemulsion (NEP) Against UVB Irradiation Inducing Photoaging in Human Dermal Fibroblast (HDF)

Anggraini Barlian*, R. Agustin, Ramadhani Eka Putra

School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

*) Corresponding author; e-mail: aang@sith.itb.ac.id
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Abstract

The skin damage induced by UV irradiation can cause photoaging as a consequence of reactive oxygen species (ROS) generation. Propolis is one of the most abundant natural product sources of polyphenols and derived from various plant resins collected by honeybees. This study aimed to determine the due administration of the propolis nanoemulsion (NEP) as an anti-photoaging by observing the concentration of intracellular ROS and lipid peroxides in human dermal fibroblast (HDF) by exposure to UVB. The cytotoxic of NEP was tested by the MTT assay, intracellular ROS was measured using the DCFDA assay, lipid peroxide products were analyzed by the TBARS assay and cell nuclei was observed by DAPI staining. PSA analysis revealed that the sizes of NEP were about 131.9 to 131 nm. The optimum concentration of NEP was 0,1µg / ml. ROS production in HDF treatment of NEP significantly decreases ($p < 0.05$). Lipid peroxide products showed the same pattern as ROS concentration. The lipid peroxide concentration in the treatment NEP significantly decreases ($p<0.05$). In conclusion, the propolis in NEP acts as an antioxidant and has the potential to reduce the production of ROS and lipid peroxides caused by exposure to UVB.

Keywords: HDF cells, nanoemulsion, propolis, photoaging, UVB, MTT, ROS, DCFDA, TBARS

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1. Introduction

Aging is a biological process that naturally happens in all living organisms. Aging process occurs in all organs of the body including skin as the outer organ which plays a role in the protection system [1]. Ultraviolet irradiation (UVR) can elevate the accumulation of free radicals within cells. The free radical is an unstable molecule that is highly reactive and seeks for its electron pair. Free radical molecule is also called reactive oxygen species (ROS) [2]. Direct and indirect UVR will activate various inductive ROS reactions and initiate the signal transduction pathway which causes skin damage and stimulate premature aging in skin due to UVR, a process called photoaging [3]. UVR, especially UVA and UVB, can penetrate through the dermal layer and cause photoaging [4].

The concept of Anti-Aging Medicine (AAM) considers aging as a prevented disease to increase human quality as people getting old [5]. Using the additive antioxidant is one of the solutions often applied to prevent or slow the aging process. One of the natural materials which contains a high antioxidant property is propolis. Propolis contains several complex natural compounds, which have potential antioxidant properties such as flavonoids and polyphenols [6]. Several studies have mentioned the role of propolis as an anti-microbe [7], anti-inflammation [8], antitumor [9, 10], and antioxidant [11]. Until now, there is no study that tests propolis as an anti-photoaging property in the human skin. In tropical country, high exposure of UVR throughout the year will increase photoaging risk in the skin.

In this study, propolis was applied in the form of propolis nanoemulsion (NEP) into human dermal fibroblast (HDF) cells as a photoaging model. The UVB irradiation treatment, which has fifth times photon energy greater than UVA, is assumed to be more harmful and can cause photoaging in skin. In this study, the role of NEP in inhibiting photoaging in HDF cells due to UVB exposure was done by ROS concentration analysis, lipid peroxide product concentration, and nucleus morphology. The objective of this research is to know the effect of nanoemulsion propolis (NEP) as an anti-photoaging compound by observing the concentration of intracellular ROS and lipid peroxide products in HDF cells after being irradiated using UVB.

2. Methods

2.1. Cell culture

Human dermal fibroblast (HDF) cells were obtained from 3 and 5 years old male's preputium tissue by a primary explant culture method. HDF cells cultured in the growth medium Dulbecco's Modified Essential Medium (DMEM, Biowest, cat. number: L0064-500) was added with 10% Fetal Bovine Serum (FBS) (Biowest, cat. number: S1810-500), 100 IU/ml penicillin-streptomycin (Gibco, cat. number: 15140148), 50µg/ml gentamycin (Gibco, cat. number: 15750060), and 2mM L-glutamine (Biowest, cat. number: X0550-100). HDF cells were grown in the 25 cm^2 flask (Iwaki) at 37 ^{0}C inside the incubator CO2 5% (Heraeus). The growth medium was replaced every 3 days. Cells, which were confluent, were subcultured into two 25 cm^2 flasks using 0, 02% EDTA, and 0, 25% trypsin (Sigma).

2.2. Propolis Extraction and Identification of Active Substance

Propolis was extracted using maceration method as has been done by Jun [6]. Extraction was done by using 70% ethanol as solvent. The solution was stirred steadily for seven days with 200 rpm speed in the dark room. Then, the solution was filtered and concentrated using rotary evaporator. The active substances in ethanolic extracts of propolis (EEP) was analyzed using Gas Chromatography-Mass Spectrometry (GCMS), and anti-oxidant activity of EEP was measured using 2, 2-diphenil 1-pichylhydazyl (DPPH assay).

2.3. Formulation and Characterization of Nanoemulsion Propolis (NEP)

Nanoemulsion propolis (NEP) formulation was done using sonication method described by Mauludin et al. [12] by adding colliphor RH40 as a surfactant, glycerin as co-surfactant, and VCO as oil phase. Surfactant and co-surfactant were stirred using a magnetic stirrer, then the oil phase and propolis were added. The next step for facilitating nanoemulsion formation was sonication using the water bath sonicator. The NEP was added with deionized water gradually and homogenized until the mixture was clear, stable, and the particle size was under 200 nm. The diameter of NEP globule was observed using Particle Size Analyzer (PSA, Delsa Nano C particle analyzer Beckmann Coulter). The existence of active substances in NEP was analyzed using Spectrophotometer Fourier Transform Infrared (FTIR) according to Butnariu & Giuchici [13].

2.4. Cytotoxic Assay

The determination of NEP concentration was done using cell viability evaluation. The cells was treated with NEP in series of concentration (0 µg/ml, 0.1 µg/ml , 0.5 µg/ml, 1 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml) with different incubation time. HDF cells were seeded in 96 well plates, with the total cell number was 5.000 cells/well and

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incubated with complete growth medium containing NEP for 24 hours, 8 hours, and 1 hour. After incubation, cells were washed with Phosphate Buffer Saline (PBS) and incubated in growth medium for 24 hours. Cell viability evaluation was done using the MTT assay. The growth medium was discarded and then added with 100µl/well complete growth medium and added with 10µl MTT (12mM). Cells were incubated for 4 hours. After 4 hours, the medium was discarded and 50µl DMSO/well was added. The solution was resuspended and incubated for 10 minutes at 37ºC. The absorbance was analyzed with ELISA microplate reader (Bio-Rad) at 550nm wavelength.

2.5. Reactive Oxygen Species (ROS) Assay

HDF cells, which were confluent, were trypsinized and seeded in the well plate. After 24 hours, cells were treated with NEP and then incubated for one hour. Cells were washed with PBS and irradiated using UVB 200mJ/cm2 (312 nm, Philips 9W/12) in PBS.

Intracellular Reactive Oxygen Species (ROS) in HDF cell culture was measured using 2', 7'–dichlorofluorescin diacetate (DCFDA kit, Abcam). HDF cells were seeded in 96-wellplates with 10.000 cells/well. Cells were added with 100µl DCFDA 20µM and incubated 37 ^{0}C for 45 minutes. Then, cells were treated with 100µl NEP 0, 1µg/ml and incubated 37 ^{0}C for one hour. After incubation, cells were irradiated with UVB 200mJ/cm2. Fluorescence intensity formed was read immediately using the fluorescence microplate reader (Thermo scientific) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.6. TBARS Assay

Lipid oxidation by ROS was measured using Thiobarbituric Acid Reactive Substances (TBARS) assay. HDF cells were seeded in 6-wellplates at the total cell number of 500.000 cells/well. Cells were treated with NEP 0, 1µg/ml and incubated 37 ^{0}C for one hour. After that, cells were irradiated with UVB 200mJ/cm2 in PBS and incubated in complete growth medium for 24 hours. MDA measurement was done with obtaining the cells using 500µl TCA 2, 5%. The cells were centrifuged for 2 minutes with 13.000 g speed. 250µl supernatant of cell lysate was added with 200µl TCA 15% and 400µl (TBA 0, 67% and 0, 01% BHT). The mixture solution was heated for 20 minutes in a water bath at 95ºC. After cooled down at the room temperature, the solution was added with 750µl buthanol and homogenized. 200µl upper phase of this solution was MDA-TBA adduct, which seemed in pink color, was transferred into 96-wellplate and the absorbance was read using a spectrophotometer at 532 nm wavelength. MDA concentration was obtained by interpolating MDA standard curve, which was resulted from linear regression, and normalized with the total amount of protein.

2.7. DAPI Staining

Nucleus morphology was observed using DAPI staining. In principle, DAPI can make a mark on DNA with binding with nitrogen base A-T in DNA. If any, changes in nucleus morphology condition indicated by an unstained region in the nucleus means that there is a damage in DNA. HDF cells were seeded in the culture dish d=3cm (Iwaki). After UVB irradiation and NEP treatment, cells were fixed using 4% paraformaldehyde (Sigma) for 15 minutes. Cells were washed using PBS. Then, the cell membrane was permeabilized with 0, 5% Triton X-100 (Sigma) for 10 minutes. Blocking was done by incubating cells in 1% Bovine Serum Albumin (BSA). Nucleus was stained by incubating cells in DAPI 1µg/ml for 40 minutes. Cells were washed using PBS and all the samples were dripped with glycerol-PBS (2:1) and closed using cover glass. Cell morphology was observed using a laser scan confocal microscope (Olympus).

3. Results and discussion

3.1. Cell Culture

HDF was isolated from preputium tissue and it was used as a research model to study photoaging. The morphology of HDF under the microscope was fibroblastic-like and adhere (Figure 1).

3.2. Propolis Extraction and Identification of Active Substance

Propolis was obtained as one of the bee (*Trigona sp*.) products that were bred in the heterogenous forest in Subang, Jawa Barat. In this study, crude propolis can be extracted to obtain extract ethanol propolis (EEP). Based on GC-MS result (Supplementary 1), EEP contains 30 different kinds of chemical compounds. Generally, active substances contained in EEP were $\pm$ 55% polyphenol and ester which has a role as an antioxidant, and the others were $\pm$ 12% essential oil, and 33% other organic compounds. The active substances which had a role in antioxidant activity were found as phenolic acid, polyphenol, and catechol. This result was supported by the study of Kurek-G´orecka *et al*. [14] which mentioned that ± 50% propolis composition was flavonoid, phenolic acid, and ester derivation.

From the DPPH result, IC_{50} score for EEP was 127.759 µg/ml and IC_{50} score for ascorbic acid was 82.443 µg/ml (Supplementary 2). With comparing this IC_{50} score, it was

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known that EEP had relative anti-oxidant potency 0,65 times greater than ascorbic acid. elative anti-oxidant potency of EEP in this research was better than Talla's research [15] that stated EEP had relative anti-oxidant potency only 0,13 times greater than ascorbic acid.

3.3. *Formulation and Characterization of Nanoemulsion Propolis (NEP)*

The optimization result showed that NEP formed from 5% VCO, 40% surfactant (Kolliphor RH40 : glycerin = 3:1), 3% EEP, and 52% aquabidest has a dark red color. Droplet NEP was measured and had a globule size in 131nm, in pH 6,8 (Figure 2).

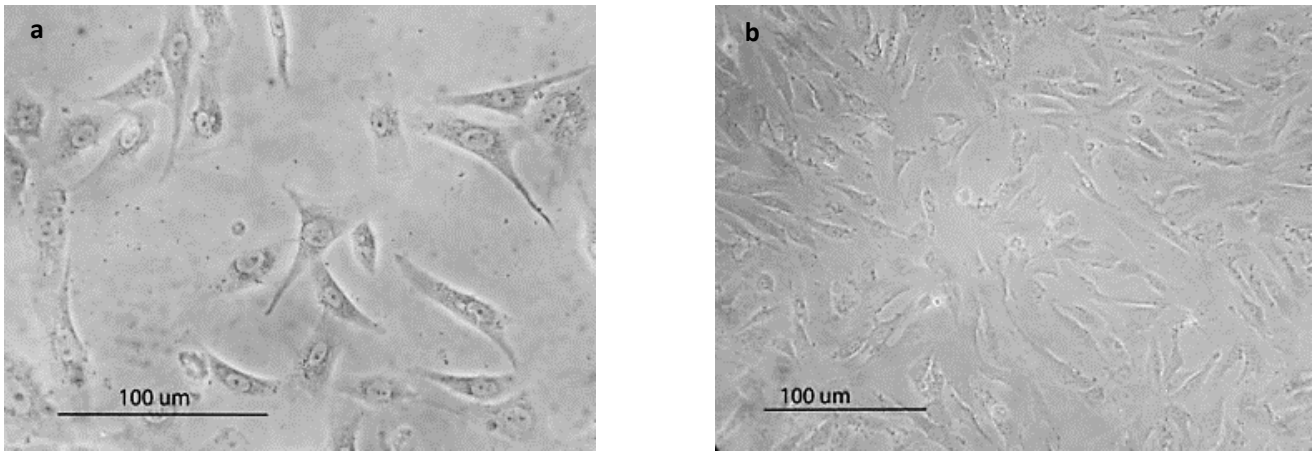

Figure 1 HDF cells from primary culture. (a) Morphology of HDF cells (magnification 400x), (b) HDF cells reached 70% *confluent* (magnification 200x).

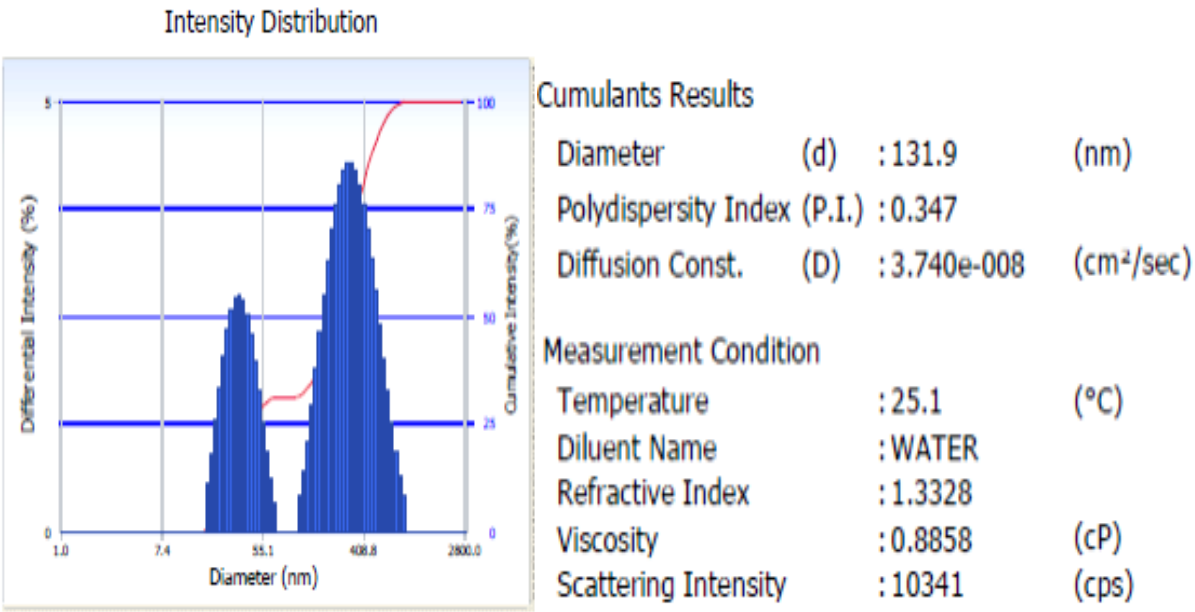

Figure 2 NEP Characterization using Particle Size Analizer

Composition analysis using FTIR (Figure 3) proved that the peak of NEP was the same as the peak of EEP. The peak of NEP and EEP at the frequency ± 2930 and ± 3300 cm^{-1} was known as the O-H group that indicates alcohol and phenol compounds. Besides that, there was also peak frequency at 1730 cm^{-1} that identified as C-O and C=O group which are ester compound and carboxilate acid. In addition, vibration in the peak of frequency ± 1300, ± 1400 cm^{-1} were

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identified as the C-H group which are alkene compounds. Another alkene compound was also found at frequency ± 1630 cm^{-1}. These findings were similar to Butnariu *et al*. [13] study which showed that NEP had frequency at 1450 and 1460 cm^{-1} that confirmed the existence of the C-H group and frequency at 1700 cm^{-1} showed C=O group as characteristic of NEP. Thus, this study confirms that the characteristic of propolis did not change while developing NEP since the chemical groups in both types of propolis were the same as shown by the FTIR analysis.

3.4. *Cytotoxicity of NEP*

Cytotoxicity of NEP was determined by measuring the cell viability with an MTT assay. NEP at 0,1-1µg/ml concentration did not cause any significant decline to cell viability ($p<0,05$). NEP concentration above 10µg/ml was toxic, therefore cell viability fell into under 50%. This phenomenon was analyzed using the toxicity parameter [16].

The incubation time after cells were exposed with NEP at these three concentrations (i.e., 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml) were 1, 6, and 24 hours before the UVB irradiation. There is no significant difference among the nine groups. NEP treatment at 0,1µg/ml concentration, which was incubated for one hour (*), showed the optimal concentration and significant difference in comparison to UVB exposure without the NEP treatment group (**) ($p<0,05$) (Figure 4).

Cells incubated in 0,1µg/ml NEP for 1 hour showed a significant increase (i.e., 12%) compared to cells in UVB irradiation without NEP treatment (Figure 5). The increase of cell viability after NEP treatment was assumed due to the inhibition of damage and cell death mechanism which affected by high ROS production within the cells. This inhibition was occurred due to the presence of polyphenol in NEP (Figure 6).

3.5. *NEP Potency as Anti-photoaging Agent*

In this study, HDF cells exposed to UVB in 200mJ/cm^2 dosage showed an increase of fluorescence intensity. This fluorescence showed the existence of ROS which is high after UVB irradiation.

Also, NEP treatment at 0,1µg/ml concentration decreased ROS production in HDF cells after being exposed by UVB irradiation. The fluorescence intensity in this treatment decreased with a score of 20%. This score showed a significant difference ($p<0,05$) compared to UVB irradiation without any NEP treatment (Figure 6). The decline of ROS production showed the NEP effect as an anti-photoaging agent in HDF cells. Active substances in EEP, i.e., polyphenol and catechin, act as an antioxidant. This antioxidant property was assumed to decrease the intracellular ROS through scavenger activity of ROS and increasing intracellular antioxidant, e.g., SOD, GPx, CAT, and other compounds. A study of Sarma et al. [17] showed a similar result. Polyphenol, which has an aromatic ring with –OH or $–OCH_3$ groups, acts as a radical scavenger by giving the electron or hydrogen atom directly to ROS [17]. The increase of antioxidant enzyme in observational groups occurred because of polyphenol and catechin activity. Lopes *et al*. [18] also mentioned that propolis can increase the antioxidant enzyme activity (e.g., SOD, GPx, and CAT) in mice's lungs which were exposed to cigarette's smoke. Thus, it can be concluded that propolis can decrease ROS production.

3.6. *The Effect of NEP on Lipid Peroxidation*

The lipid membrane component in the cell is vulnerable to damage, mainly by irradiation [19]. This study showed that UVB irradiation to HDF cells increased the lipid peroxide product and NEP decreased lipid peroxide product that has been formed (Figure 7).

Polyunsaturated fatty acid (PUFA), which was contained in the cell membrane, is very sensitive to ROS. Hydroxyl radical (·OH) compound could cause peroxidation in PUFA. The lipid peroxidation process has been mentioned by Sharma *et al*. [17] and such mechanism was also assumed to happen in this research. Lipid peroxidation was started from initiation reaction by ROS activation. This ROS react with methylene group from PUFA formed lipid peroxy radicals and hydroxyperoxide. Malonialdehyde (MDA) is the final product from fatty acid peroxidation in phospholipid membrane and can potentially damage the cell membrane. In this research, the formed MDA can be measured as a lipid peroxide, which was formed due to UVB irradiation.

The concentration of lipid peroxide in NEP treatment reached a score of 18%, showing a significant difference ($p<0,05$) compared to UVB treatment only. The fall of lipid peroxide product was assumed due to the existence of active substances in EEP such as polyphenol and catechin which have a role in initiating process by inhibiting ROS production, therefore it affected the following reaction in lipid peroxide formation. It was also supported by the statement from Sharma *et al*. [17] that polyphenol inhibited ROS and lipid peroxide by blocking radical lipid alkoxyl. In this study, propolis has a role in inhibiting ROS and made lipid peroxide concentration dropped. This was supported by Silva *et al*. [20] who stated that propolis contained polyphenol which is very effective in inhibiting lipid oxidation. This indicated that polyphenol compound in propolis has a role as lipid protection of cell membrane. Thus, EEP formulated as NEP has potential as a lipid peroxidation inhibitor by scavenging ROS in initiating the process of lipid peroxidation due to UVB irradiation.

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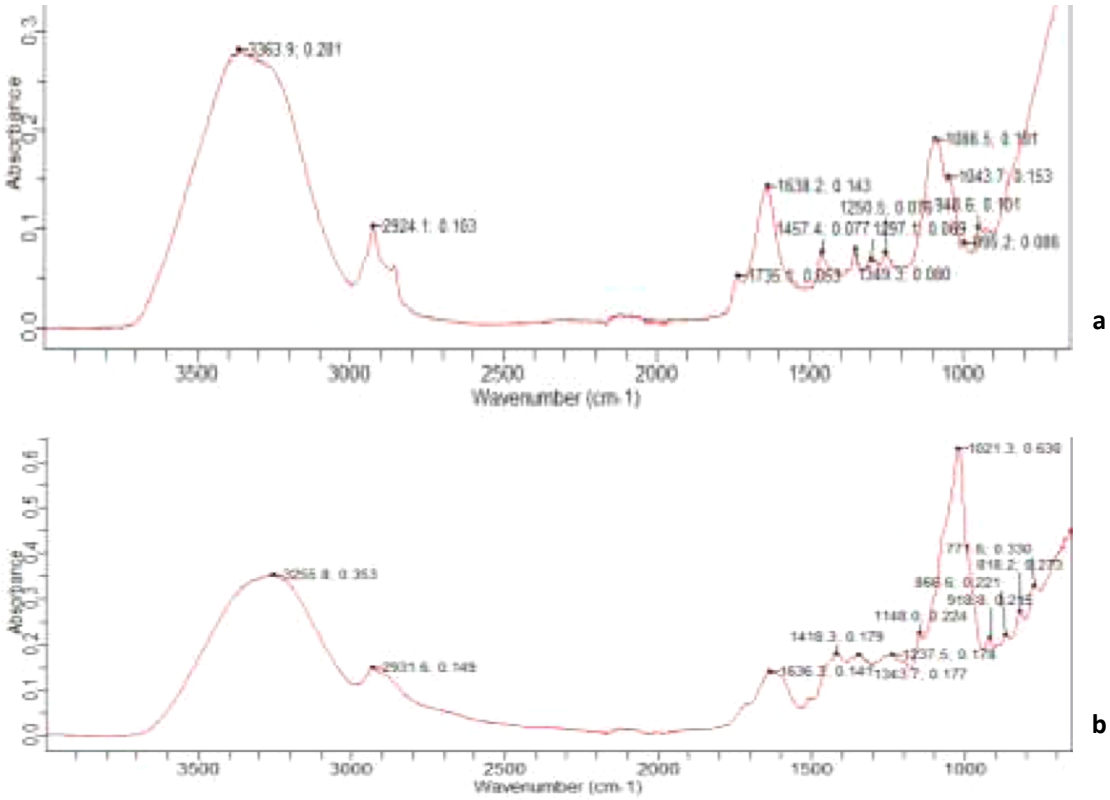

Figure 3 FTIR Chart that showed composition of chemical groups at (a) NEP and (b) EEP

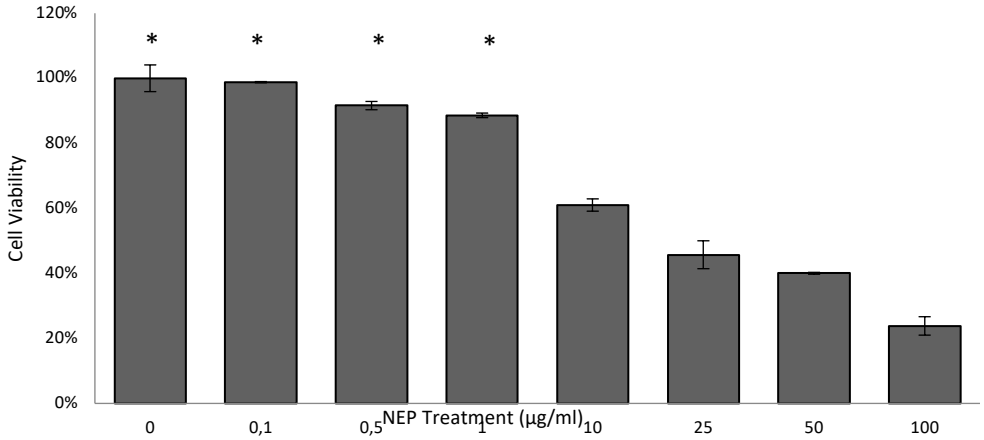

Figure 4 HDF cell viability percentage after NEP treatment in different concentration for 24 hours. NEP exposed in 0,1-1µg/ml (*) did not cause any significant effect in compare to control group (HDF cells without any treament(*)).

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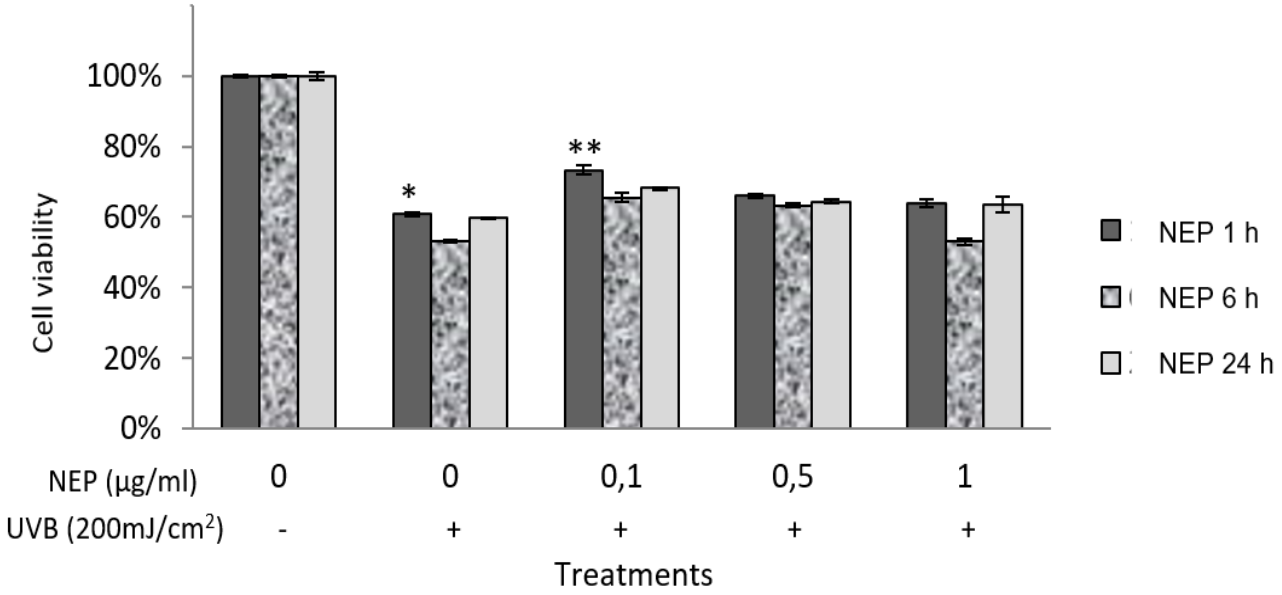

Figure 5 Percentage of cell viability after NEP treatment in different concentration (0,1-1µg/ml) each group for 1, 6, and 24 hours. NEP treatment in 0,1µg/ml concentration for 1 hour (**) showed a significant difference ($p<0,05$) in compare to HDF cells after UVB exposure group (*).

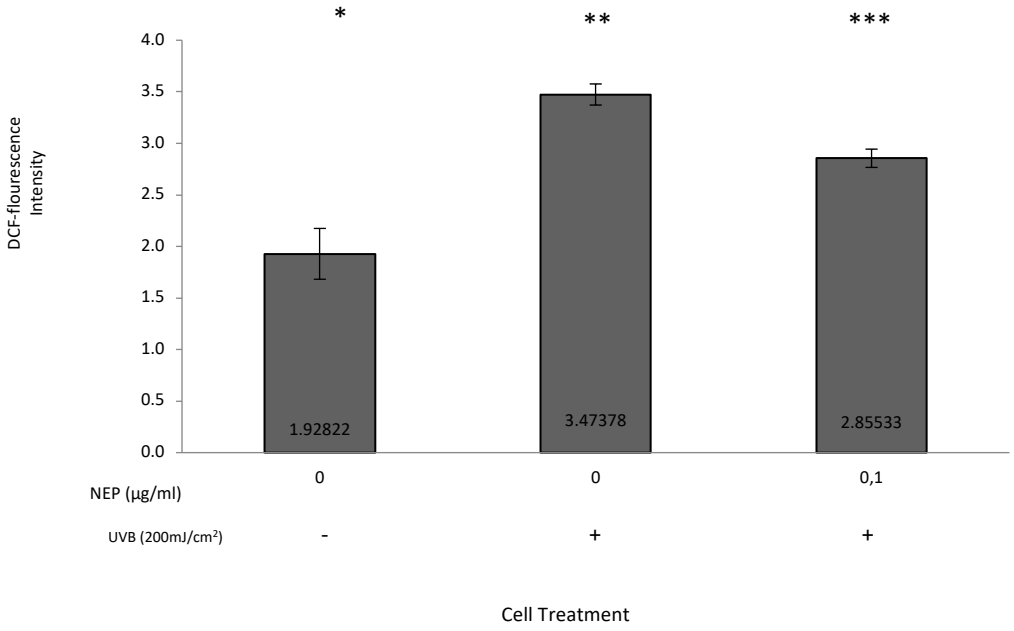

Figure 6 Fluorescence intensity within HDF cells that represented intracellular ROS after NEP 0,1µg/ml treatment for 1 hour. NEP exposure affected the descline of ROS products within HDF cells (***) in comparison with HDF cells which irradiated with UVB without NEP treatment(**).

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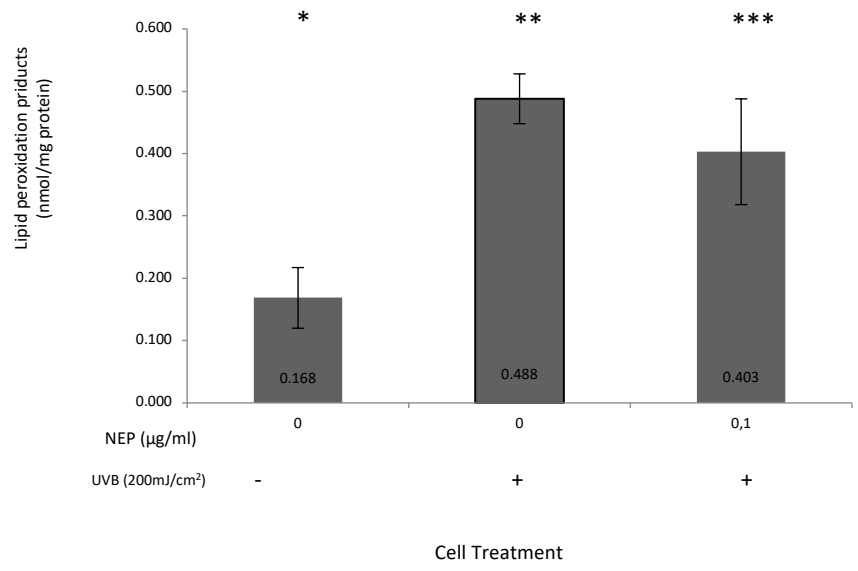

Figure 7 Concentration of lipid peroxide product in HDF cells after NEP 0,1µg/ml treatment for 1 hour. NEP treatment affected the decline of lipid peroxide product in HDF cells (***) in compares to HDF cells were only treated by UVB irradiation (**).

3.7. The Effect of NEP on HDF Nucleus Damaged by UVB Irradiation

The change of nucleus morphology was indicated by the unstained region after DAPI staining, marking the damage in the DNA. In this research, UVB exposure with 200mJ/cm^2 dosage can change the nucleus morphology (Figure 8). DNA damage due to UVB irradiation has been well-known because of the photon energy from UVB that can bind directly with DNA and produce dipyrimidine photoproduct, such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) photoproducts (6-4PPs). Dipyrimidine photoproduct is known for its ability to change DNA conformation by forming kink because of the existence of lesion in DNA structure which cause irregular DNA structure [21]. Dipyrimidine photoproduct occurs because of the unexpected binding between nitrogen base in DNA, especially thymine and adenine. Thymine does not bind the adenine, therefore DAPI in the observational groups can not bind A-T bindings in DNA. In this study, there was an unstained region by DAPI (Figure 8.b). However, this study did not analyze DNA damage using a specific marker to evaluate the damage directly. Straface *et al*. [22] mentioned that UVB irradiation with 200mJ/cm^2 dosage into fibroblast cell culture caused DNA damage. DAPI staining in Straface study showed a similar result with this study, which was the presence of an unstained region by DAPI inside the nucleus after UVB irradiation. In addition, Straface *et al*. [22] evaluated the same phenomenon by doing a spesific Hoechst stain which also showed an unstained region. Those were showed as fluorescent spots in the nucleus. Straface *et al*. [22] also stated that this condition was correlated with the unique reorganization activity after cells being exposed by UVB irradiation and indicated the cellular senescence.

Nucleus morphology in HDF cells after NEP 0,1µg/ml treatment also showed an unstained region (Figure 8.c). In this study, there was no quantified calculation. However, the unstained region in NEP treatment seemed fewer than in UVB irradiation treatment. It was assumed because NEP had a role in inhibiting nucleus damage indirectly. Polyphenol and catechin in NEP have a role as anti-oxidant that can inhibit ROS which can impair DNA indirectly. The research of Stojilijkovic *et al*. [23] also stated that nucleus damage due to UVB irradiation by an indirect pathway through ROS formation can damage the nucleus.

ROS was inhibited by the anti-oxidant properties in NEP. In addition, it was occurred because EEP, as one of the components of NEP, had absorbance spectrum activity to UV. The main substance of EEP is polyphenol, and the biggest part of natural polyphenol is known as a pigment that can absorb UV irradiation. Propolis has absorbance spectrum activity to UV with maximum absorbance at 290 nm wavelength that includes UVB wavelength (290-320nm) [24]. In this study, NEP was assumed as a photoprotective agent in reducing DNA damage in the nucleus due to UVB irradiation.

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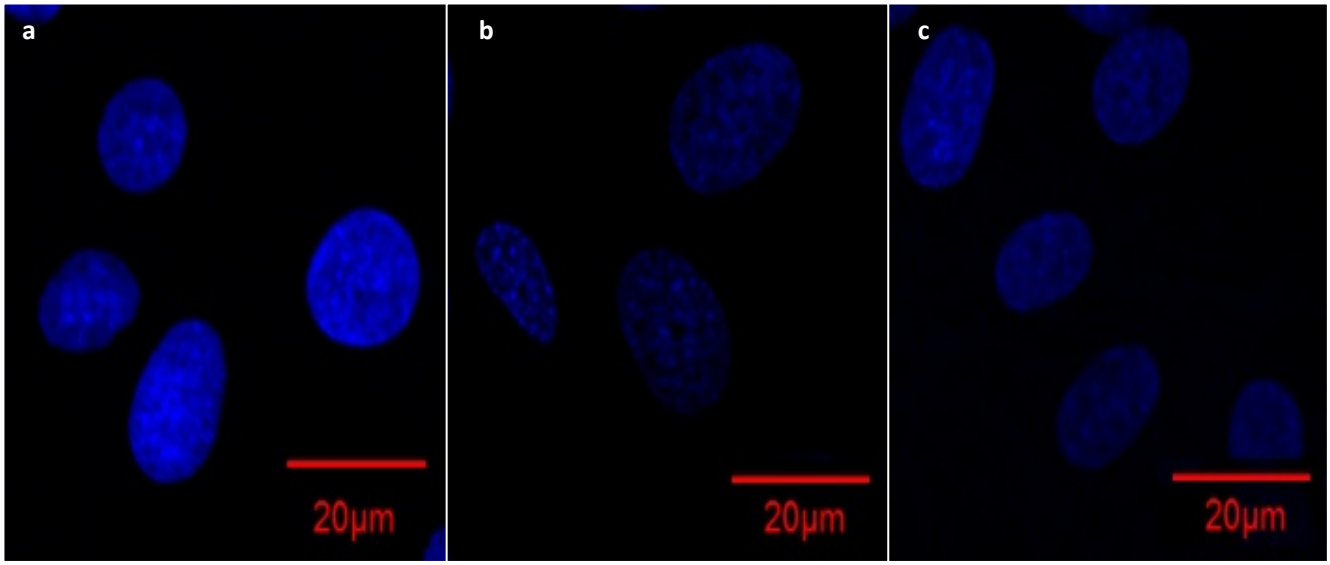

Figure 8 Nucleus morphology of HDF cells which was observed by DAPI staining. (a) normal control; (b) 200mJ/cm^2 UVB treatment; (c) NEP 0,1µg/ml treatment followed by 200mJ/cm^2 UVB irradiation.

4. Conclusion

UVB irradiation at 200mJ/cm^2 can stimulate stress condition to HDF cells, causing an increase of ROS production. NEP exposed pre-treatment can reduce stress condition due to UVB. There was a decline in ROS production until 20%. UVB irradiation at 200mJ/cm^2 can rise the lipid peroxide in HDF cells. NEP exposed pre-treatment can reduce lipid peroxide until 18% due to UVB. Polyphenol and catechin contained in propolis have a role as antioxidants which was given in NEP formed in reducing stress due to UVB irradiation.

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Aging Process in Dermal Fibroblast Cell Culture of Green Turtle (*Chelonia mydas*)

Anggraini Barlian*, Yemima D. Riani

School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

*) Corresponding author; e-mail: aang@sith.itb.ac.id
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Abstract

Senescence in green turtle is an interesting process to study because until now no clear explanation has been established about senescence at cellular or molecular level in this species. One of common markers used for detecting senescence is telomere shortening. Reduced telomerase activity may also cause senescence. The aims of this research are to establish and identify dermal fibroblast cell culture from green turtle and also to compare telomere length and telomerase activity from the cells subculture 5 and 14. Cells were identified with Rabbit Anti-Vimentin Polyclonal Antibody and Goat Polyclonal Antibody using confocal microscope. Telomere length was obtained using TeloTAGGG Telomere Length Assay (Roche), while telomerase activity was obtained using TeloTAGGG Telomerase PCR ElisaPlus. Primary cell culture from green turtle skin showed fibroblastic morphology and immunocytochemistry results using vimentin antibody proved that the culture was fibroblast cell. Measurement of telomere length and telomerase activity showed that telomere length and telomerase activity of subculture 14 was greater than subculture 5. However, based on morphology, green turtle fibroblast skin cell culture showed senescent morphology. Therefore, possible aging mechanism that the green turtle fibroblast skin cell culture underwent did not go through both telomere shortening and reduced telomerase activity.

Keywords: cell culture, *Chelonia mydas*, telomerase activity, telomere length, senescence.

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1. Introduction

Green turtle (*Chelonia mydas*) is one of the most popular long-lived animals whose age may exceed 100 years [1, 2]. Aging in *C. mydas* is still unknown because early studies showed no mortality advances yet increasing reproductive capacity [3]. The study of aging in *C. mydas* is important for further comparison with mammals in the biomedical field. However, since 1999, IUCN recorded *C. mydas* as one of the endangered species, therefore sacrificing this animal for research must be minimized. One solution for this issue is the establishment of *C. mydas* cell culture. The establishment of new cell culture from primary explants may produce one dominant cell type dependent on medium, growth factor, and tissues collected from the donor. Therefore, cell type must be analyzed first before the aging process can be analyzed further. As cells replicate in culture, the cell will experience senescence caused by telomere shortening. Telomeres are repeated sequences at chromosome end and will become shortened at cell replication because of end-replication problem [4]. DNA bases can only be added from hydroxyl 3' end, so replication of lagging strand can only be processed by the presence of RNA primer. Once replication reaches the end of a chromosome, RNA primer at the 3' end degraded and no DNA base can be added at this end. This telomere shortening phenomenon is one of the main causes of replicative senescence which leads to an estimation of Hayflick limit, number of which cells can be passage until cells stop divide, and finally undergo apoptosis [5]. As cells replicate and chromosomes shortened, chromosome fusion, break, and damage could happen to DNA. Thus, cells will activate p53 protein to arrest the cell cycle in the late G1 phase before cells may show senescence phenotype such as decreased protein degradation, housekeeping enzyme expression, energy production, and increased ROS level (reactive oxygen species) [6]. This phenotype may lead to a decrease in normal cell function, after which cell may undergo apoptosis. The presence of telomerase, a ribonucleoprotein, prevents telomere shortening because telomerase adds extra TAGGG base to telomere end [7]. In mammals, telomerase is no longer expressed in adult tissues [8], except in cancer [9] and stem cells [10]. Hence, analysis of telomere length and telomerase activity could be markers of aging in cell culture. Since *C. mydas* may not have a regular aging process such that is found in mammals, we hypothesize that telomere length and telomerase activity in dermal fibroblast cell culture at subculture 14 is higher than subculture 5.

2. Methods

2.1. Cell culture

Explants for cell culture were biopsied from a 2-year old *C. mydas* hindleg skin. Explants were washed in PBS and then cultured in Leibovitz-15 medium (Sigma, cat. number: L5520), supplemented with 100 U/mL (P/S) (Sigma, cat. number: P4333) and incubated at 30 ± 1 ºC in incubator (BlueM). Medium was changed every three days and subculture was performed by using 0.2% EDTA and 0.02% trypsin (Sigma, cat. number: T4049).

2.2. Immunocytochemistry

Cells at passage 2 were plated on cover glass. After cells reached confluent, cells were fixed in methanol/PBS series. 0.05% PBST was used to increase cell membrane permeability. Cells were hybridized with Rabbit Anti-Vimentin Polyclonal Antibody (ab-45939, Abcam) with concentration 4:1000 in PBS. After that first hybridization, fixed cells were blocked with 0.3% BSA-PBS. Fixed cells were hybridized further with Goat Polyclonal Anti-Rabbit IgG-FITC (ab-6717, Abcam) 4:1000 and mounted in glycerol/PBS 1:9. Cells preparation was then analyzed under confocal microscope (Zeiss, LSM 710) with maximum intensity.

2.3. Telomere Length Assay

Cells at subculture 5 and 14 were harvested using 0.02% EDTA and 0.2% trypsin. DNA isolated from these two subcultures with High Pure PCR Template Preparation Kit (Roche). Telomere length analysis was performed with TeloTAGGG Telomere Length Assay (12209136001, Roche). As much as 1µg DNA was restricted with HinfI/RsaI, then separated in 0.8% agarose (Sigma) gel electrophoresis with 1x TAE Buffer for 4 hours at 50 V. DNA was transferred to nylon membrane (11417240001, Roche) with capillary transfer method. Membrane hybridized with telomere probe and *DIG Easy Hyb Granules* was then reacted with *Anti DIG-AP* and substrate solution. Membrane was then analyzed with chemiluminescence assay and transfered to X-Ray film (Fujifilm). Telomere length was measured with ImageJ software.

2.4. Telomerase Activity Assay

Telomerase activity assay was done with TeloTAGGG Telomerase PCR ElisaPlus (120113789001, Roche). Protein from cells at subculture 5 and 14 was extracted with Lysis Buffer and centrifuged at 16.000 x g for 20 minutes. Protein concentration was measured with Bradford method. Telomerase was then added with DNA base to biotin-labeled synthetic primer with Telomeric Repeat Amplification Protocol (TRAP Assay). Furthermore, amplification result was hybridized with hybridization buffer T and hybridization buffer IS microplate module for analysis based on spectrophotometry. The result was analyzed in ELISA

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microplate reader (BioRad) at wavelength 450 nm and 590 nm.

3. Results and discussion

3.1. Cell Identification

Cells cultured from *C. mydas* skin showed fibroblastic morphology which has spindle shaped-like structure with long cytoplasmic protruding. Cell staining with vimentin antibody and analysis using confocal microscope showed positive result which ensured us the cell type of *C. mydas* skin cell culture is fibroblast (Figure 1).

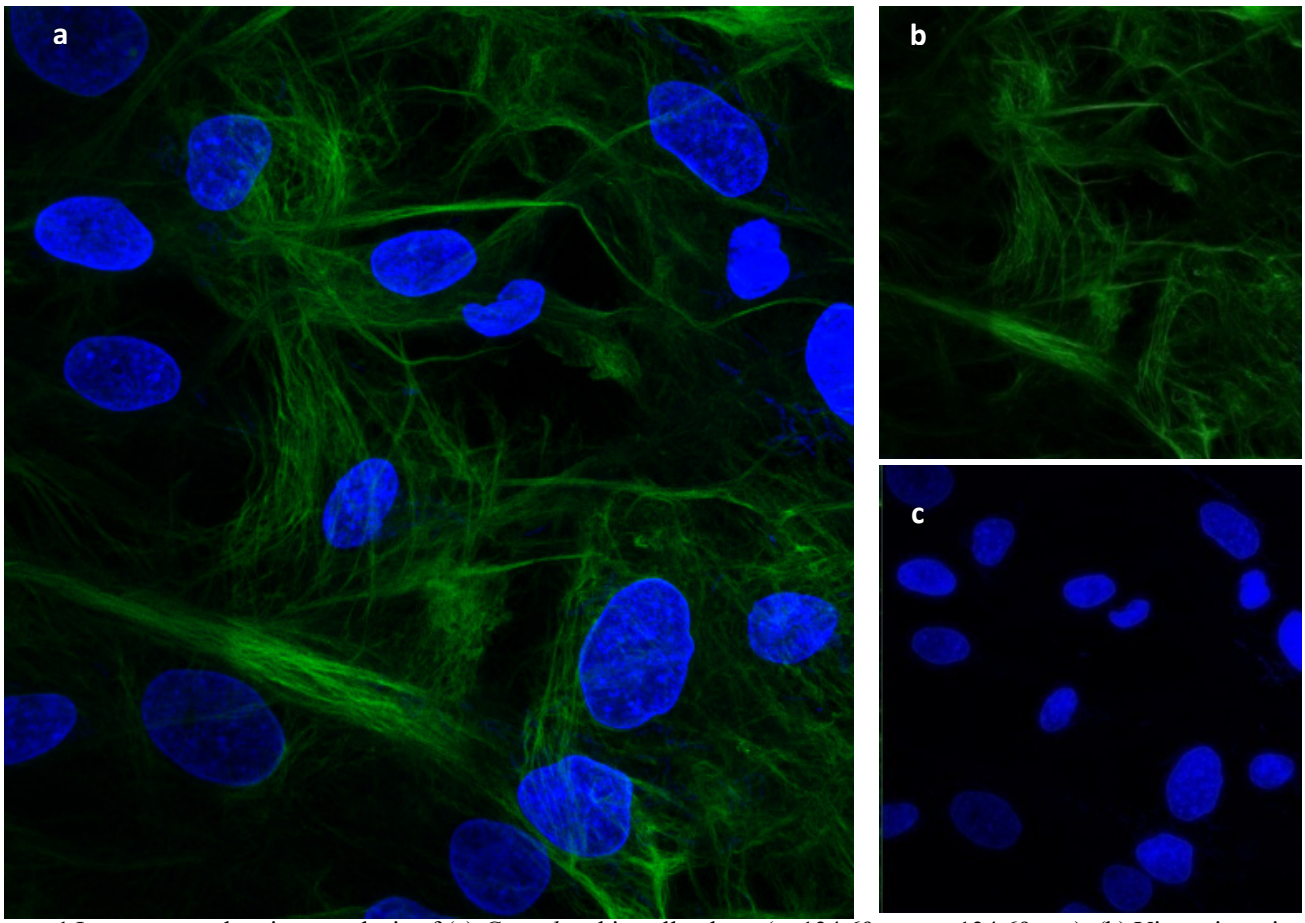

Figure 1 Immunocytochemistry analysis of (a) *C. mydas* skin cell culture (x: 134.69 µm, y: 134.69 µm). (b) Vimentin stained with rabbit anti-vimentin antibody and goat anti-rabbit IgG-FITC.(c) Nuclei stained with DAPI

Although the primary source of tissue culture, skin biopsies, is a mixture of various kinds of cells, skin cell cultures of *C. mydas* was eventually dominated by a particular cell. In terms of morphology, it can be concluded that the type of cell that is successfully cultured were fibroblasts. The FBS used in this research contained abundant growth factors including bFGF (basic fibroblast growth factor) that will support fibroblast growth [11].

Fibroblasts are interstitial cells which build connective tissue. Fibroblasts were derived from mesenchymal cells and produce extracellular matrix such as collagen I and III, glycosaminoglycans, elastic fibers, and glycoproteins. Therefore, the role of fibroblast cells in the skin tissue is important if it is associated with the aging process. Reduced ability of skin fibroblasts to synthesize extracellular matrix collagen I and III will be associated with the appearance of wrinkles on the skin, which is the most particular symptom in the skin aging process [12]. Fibroblasts as a proponent of the connective tissue must be able to withstand mechanical stress. The ability of fibroblasts to migrate and have been known to withstand mechanical stress is determined by vimentin [13]. Vimentin is type III intermediate filament and is used as a marker of fibroblasts [14].

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3.2. *Telomere Length Analysis*

Telomere length in *C. mydas* is greater than telomere length in mammals [15]. Telomere length in human ranges from 10 to 15 kbp [16]. Marker used in this assay ranged from 0.8 to 21.2 kbp. However, Figure 2 showed that *C. mydas* telomere length is beyond 21.2 kbp. Therefore, measurement of telomere length was not accurate. However, the increase or decrease in telomere length can still be estimated.

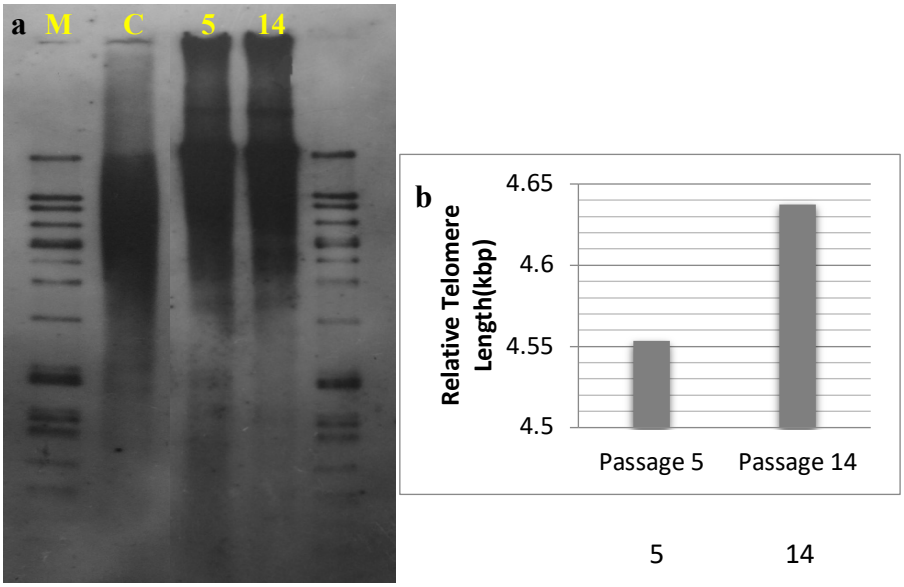

Figure 2 Telomere length in cultured skin fibroblasts of green turtles (a) The results of the analysis of telomeres in skin fibroblasts cultured from green turtle (M = marker, C = control DNA (human dermal telomere), DNA subculture 5 = 5, 14 = DNA subculture 14). (b) Comparative graph of telomere length on DNA subculture 5 and 14

Figure 2.b showed that telomere length at subculture 14 was greater than subculture 5. This result was different than what is commonly found in human. In human fibroblast cell culture, telomere length decreases as the subculture increases [17]. In human endothelial cells culture [18], telomere shortening can be observed between passage 4 and 13.

No-telomere shortening phenomenon is also found in *Emys orbicularis* [19], which shows that the length of telomere from embryo blood cells was no different with the adult organism's. In *Dermochelys coriacea,* another member of Cheloniidae family, a study also shows similar results in which telomere shortening does not occur in blood cell embryo or adult organism [20]. However, conflicting results were found in *Caretta caretta*, which comes from the same family with *Chelonia mydas*. Telomeres in blood cells and adults epidermis of *Caretta caretta* experience telomere shortening with age. It can be concluded that the relationship between age and telomere length could be tissue and species-specific.

3.3. *Telomerase activity analysis*

Telomerase activity assay result in *C. mydas* aligns with the telomere length result, which is higher in subculture 14 than 5 (Figure 3). This phenomenon is also different if we compare the result to human. In human, telomerase activity cannot be found in adult tissues. Based on telomere length and telomerase activity, our *C. mydas* dermal fibroblast cell culture from subculture 5 to subculture 14 did not undergo senescence.

Previous research [15] showed that telomere elongation occured and increased telomerase activity in several organs of newly hatched, as compared to 7-month old, *C. mydas*. In this research, telomere length and telomerase activity in cells at subculture 14 was higher than in subculture 5 (Figure 2 and Figure 3). This consistent phenomenon occurred both in vivo and in vitro may conclude that *C. mydas* fibroblast skin cell culture can be used as a good material for further research on the aging process.

In all human organs, except reproductive organs such as testis, telomerase activity does not exist after birth [8], as well as in cultured condition [21]. Allegedly green turtles have a special mechanism that does not exist in human to increase telomere length and telomerase activity with age. The phenomenon of increased telomerase activity with age

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occurs not only in *Chelonia mydas* but also in several other species of the order Testudinae, such as in *Chrysemis picta* and *Chelydra serpentine* [22]. *Chelydra serpentine* cell culture indicated the presence of telomerase activity in subculture 157 and continued to increase until subculture 191. Thus, in the future, green turtle can be developed as an animal model for the aging process in the field of biomedicine.

3.4. Morphology analysis

Morphology of the cell differs between subculture 5 and 14 (Figure 4). Cells at subculture 14 had enlarged nucleus, more vacuoles, and showed multinuclei structure. This result showed some phenotype of aging cells and also cells doubling time was longer as the subcultures get higher.

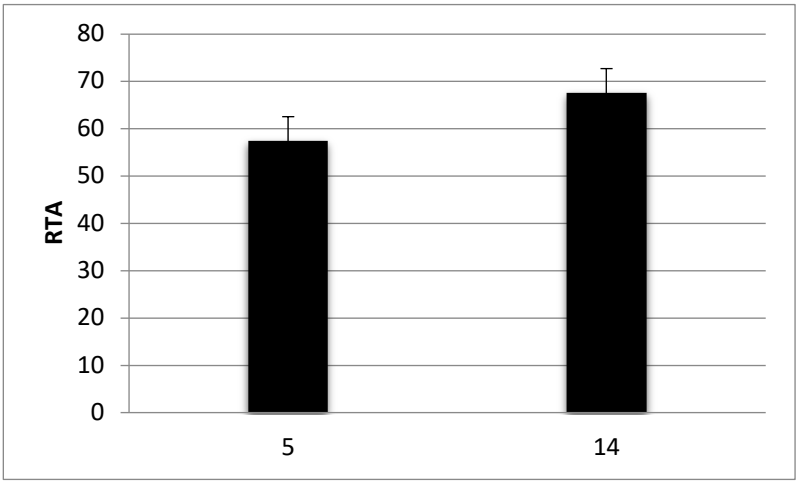

Figure 3 Comparative graph of telomerase activity in 5 and 14 subcultures. Relative telomerase activity (RTA) subculture 14 is higher than 5

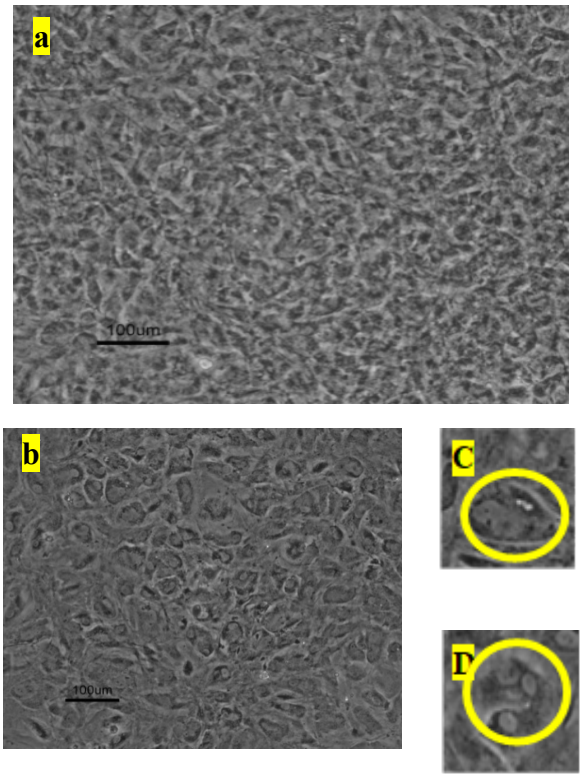

Figure 4 Cell morphology subculture 5 and 14. (a) *C. mydas* skin fibroblast cell culture subculture 5 (45x). (b) *C. mydas* skin fibroblast cell culture subculture 14 (45x). (c) Enlargement of vacuoles in the cell at 14. (d) Multinuclei cell at subculture 14

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Based on morphology analysis, it was shown that *C. mydas* fibroblast skin cell culture underwent aging. Cells in subculture 14 are relatively larger than subculture 5 and also have epithelial-like morphology. According to Baba and Catoi [23], enlargement of cell size and changes of shape can be caused by two things, the cells had started to age or cells transformed into cancer cells. Characteristics of transformed normal cells into cancer cells is increasing the size of the nucleus, growing ratio of nucleus and cytoplasm, irregular cell shape, formation of multilayer cells, and increasing the rate of cell proliferation. Because cells in this research did not form multilayer nor increasing the rate of proliferation, therefore cell culture in this research presumably underwent aging. According to Phipps *et al.* [24], cells that undergo aging may increase in size, nucleus, nucleoli. Furthermore, cell phenotypes that were abundantly occurring in this cell culture and strengthening the hypothesis were multinuclei formation, increasing the number of vacuoles, as well as the increasing number of microfilaments and decreasing rate of proliferation. Characteristics that occurred in this research might be due to the increased amount of RNA and protein in cells. Protein and RNA degradation rate might also be decreased while transcription and translation activities still exist. Cells will be held in the G1 phase so that the cells size continues to increase without undergoing fission. This process may lead to the formation of the enlarged vacuoles and multinuclei. In conclusion, based on the morphology, it is observed that cells underwent aging. However, based on telomere length and telomerase activity analysis, aging did not occur because telomeres and telomerase activity increased as subcultures go higher. *C. mydas* dermal fibroblast cells aging process could be induced by stress through cell culture conditions, but not through the telomere shortening. Two main pathways that might occur in cultured dermal fibroblasts of green turtles which also occur in mammalian cells is through the p53 pathway and pRB [25].

p53 pathway can be activated through telomere shortening and increased ROS (reactive oxygen species). However, in this study, telomere shortening in *C. mydas* dermal fibroblast cell culture did not occur, thereby supposedly cells underwent aging through ROS-induced p53 pathway. ROS accumulate in cells due to incomplete redox reactions in the mitochondria (mitochondrial dysfunction) as well as environmental influences [26]. In addition, due to mitochondrial dysfunction, it is known that the amount of ROS in cells is also enhanced by the activity of Ras (oncogene) [27]. ROS causes DNA damage that triggers DNA damage response which induced p53 expression, which consequently render the cell cycle to be held in G1 phase.

Aging in cultured skin fibroblasts of green turtles can also occur through the pRB. pRB pathway is activated by the p16 cell cycle regulatory proteins. P16 protein is induced by stress stimuli experienced by *C. mydas* fibroblast skin cell culture as activated Ras by ROS and suboptimal culture conditions. pRB activation would lead to a reorganization of chromatin into heterochromatin. This means that proteins that play a role in inducing cell cycle sustainability such as G1/S cyclin and G1/S CDK (cyclin dependent kinase) could not be expressed [25]. Consequently, cells are arrested at G1 phase and cause enlargement of cell size as observed in cells at subculture 14.

4. Conclusions

In this study, we suggested that dermal fibroblast of green turtle experienced aging that is caused by stress from culture conditions that give rise to the increase of ROS in cells. Increasing amount of ROS in the cells of green turtle skin fibroblasts might induce p53 pathway and aging through the PRB. To prove the aging pathway experienced by dermal fibroblasts cell culture of green turtle, further study through the analysis of p53 and pRB gene expression is needed.

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Production of Bioethanol and Crude Cellulase Enzyme Extract from Napier Grass (*Pennisetum purpureum* S.) through Simultaneous Saccharification and Fermentation

Taufikurahman*, Sherly

School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

*) Corresponding author; e-mail: taufik@sith.itb.ac.id
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Abstract

Napier grass (*Pennisetum purpureum* S.) has been recognized for its high amounts of cellulose and hemicellulose which can be utilized for bioethanol production. Bioconversion of Napier grass to bioethanol can be performed by filamentous fungi. A fungi, *Neurospora sitophila*, can synthesize and secrete hydrolytic enzymes to breakdown the cellulose and hemicellulose into various monomeric sugars and simultaneously convert it to bioethanol. This study investigated the effect of Napier grass substrate concentrations and cultivation time on cellulase enzyme and bioethanol production by *N. sitophila*. The pretreatment of Napier grass was carried out using 1.5% (w/v) NaOH solution for 120 hours at 30 °C. After the pretreatment process, simultaneous saccharification and fermentation was conducted at 30 °C; pH 6.8-7.0 and agitation speed 130 Rpm with various of Napier grass substrate concentrations (10, 20 and 30 g l^{-1}) and cultivation period within 24; 48; 72; 96 and 120 hours. The results show that the highest enzyme activity is 0.28 FPU/ml which was achieved at 120 hours of cultivation and 20 g/L substrate concentration of Napier grass. The highest ethanol content was obtained by 96 hours of simultaneous saccharification and fermentation (1.25 g l^{-1}) using 20 g l^{-1} substrate concentration and the maximum ethanol yield is 0.30 g/g cellulose conducted at 30 g l^{-1} substrate concentration and 96 hours of fermentation.

Keywords: Bioethanol, Enzyme Activity, Neurospora sitophila, Napier grass, Simultaneous saccharification, Fermentation

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1. Introduction

Napier grass (*Pennisetum purpureum* Schumach) is a species of perennial tropical grass with fast-growing characteristics and low input requirements (e.g., low nutrient, fertilizer, and water requirements). The annual productivity can reach about 78 tons of dry weight/ha [1].The Napier grass also contains high sugars, comprising 30-37% cellulose, 20-31% hemicellulose, and 8-21% lignin [2]. The high percentage of cellulose and hemicellulose with low lignin made it as an attractive raw material for bioethanol production. Moreover, Napier grass does not compete with other food crops therefore it is reducing the food versus energy competition.

Napier grass can be converted to bioethanol by conducting several steps include pretreatment, hydrolysis, and fermentation. The pretreatment aims to breakdown the chemical bonds of lignin and cellulose and removes the lignin. The pretreatment mainly involves chemical processing (alkali or acid pretreatment) to reduce the crystallization of lignocellulose and to increase the specific areas of cellulose for enhance the process of hydrolysis [3]. Hydrolysis can be conducted using either acid or enzymatic hydrolysis. Hydrolysis using acid is reportedly not friendly because in general, the process involves an explosion to high temperatures and can generate furfural compounds that can inhibit the fermentation process [4]. Recently, enzymatic hydrolysis is known as an environmentally friendly and safe method. The enzymatic hydrolysis can be performed in separate hydrolysis and fermentation (SHF) or in the process of simultaneous saccharification and fermentation (SSF). SHF process has the main advantage to perform the saccharification and fermentation under optimal condition. However, the degrading enzyme could be affected by accumulation of high glucose obtaining from saccharification process. Correspondingly, SSF technology was developed in recent years because it enables a reduction in investment costs, saves energy and achieves higher ethanol productivity by reducing the inhibition of end-products compared to separate hydrolysis and co-fermentation [5].

Numerous of filamentous fungi are known to degrade complex substrates of lignocellulose into various monomeric sugars and simultaneously convert it to bioethanol. With this promising characteristic, one of the filamentous fungi, *Neurospora* sp. has been used for bioethanol production. The fungus has ability to secrete enzyme for cellulose and hemicellulose hydrolysis as well as enzymes to produce bioethanol [6]. However, the enzyme activity and bioethanol production is proportional with some parameters such as substrate concentration and fungi cultivation period. This work is aimed to study effects of parameter and selecting the best condition for bioethanol production from Napier grass using *Neurospora* sp.

2. Materials and Methods

2.1. Microorganism N. sitophila.

Stock cultures were cultivated on dextrose potato agar at room temperature for 3 days. The resulting conidia (spore) suspension were then calculated in a haemocytometer and achieving about 10^6 spores per milliliter. Afterward the spore suspension was transferred into liquid media (Potato Dextrose Broth or PDB) by scraping the sporulating colonies and suspended in sterile distilled water containing 0.85% (v/v) Tween 80 as wetting agent and was incubated at room temperature for 3 days on a benchtop orbital shaking at 120 Rpm. The culture was used as inoculum for bioethanol and cellulase production in the submerged fermentation.

2.2. Napier grass (Pennisetum purpureum S.)

Napier grass seedlings were obtained from CV Bintang Tani, Bogor, West Java, Indonesia. Then Napier grass was cultivated in screen house at ITB Jatinangor and harvested with the growing age of 3 months. The grasses was washed and dried by shade drying. Dried Napier leaves was milled using a blender and then sheaved to obtain the particle size of +35/-60 mesh.

2.3. Neurospora minimal medium

The growth medium for *N.sitophila* under SSF process using its minimal medium which is recommended by National Collection of Industrial Microorganisms (NCIM) as optimum medium for initial revival or subculture [7]. The medium consisted of the following mineral medium:(in g L^{-1}) KH_2PO_4 2,.0; $NH4NO_3$ 2.0; $MgSO_4.7H_2O$ 0.5; NaCl 0.1; $CuCl_2$ 0.0001; $ZnCl_2$ 0.0002; $MnCl_2$ 0.00002; $FeCl_3$ 0.00002; $Na_2MoO_4.2H_2O$ 0.000002; H_3BO_3 0.000001; and biotin 0.000001, supplemented with 0.2% wt glucose. The pH was adjusted to 6.8-7.0.

2.4. Alkaline pretreatment methods

Twenty grams of Napier grass powder was pretreated in 400 ml of 1.5% wt NaOH solution (ratio 1:20) at room temperature for 120 hours. Subsequently, the treated Napier grass were filtered, washed, sterilized, and dried at 105°C [8] was subjected to further SSF process.

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2.5. Simultaneous saccharification and fermentation (SSF) using N. sitophila.

Simultaneous saccharification and fermentation was carried out in a 250 ml flask with various substrate concentrations of Napier grass (10 g l^{-1}; 20 g l^{-1} and 30 g l^{-1}). For 10 g l^{-1} of substrate concentration, one gram of pretreated Napier grass was put into the flask. Then, the substrate was mixed with 90 ml of *N.sitophila* (90% total volume). The mixture was sterilized in a water bath at 80 °C for an hour. After sterilization, 10 ml of PDB broth containing fungus suspension was inoculated aseptically to the flask, resulting 100 ml in total. The pH of each culture was adjusted until 6.8-7.0. The flask were enclosed by cotton plugged to release the carbon dioxide produced. The culture was shaken (130 Rpm) and incubated at room temperature for 72 h in aerobic condition. After 72 hours of cultivation, the agitation was stopped. The culture was harvested periodically over 24 h and analyzed for the fungus growth, substrate availability, enzyme activity and fermentation products. Each flask was harvested everyday to represent an experimental point. All treatments were conducted in triplicate.

2.6. Quantification of fungal biomass

The mycelial biomass of the fungal in the culture was harvested by filtration through filter paper, washed with stipulated hot water to separate the mycelia and the substrate, then dried at 105 °C overnight. The dried residues were weighted and recorded. Every flask was harvested to represent an experimental point. Specific growth rate and doubling time of fungal were determined in log phase.

The specific growth rate (μ) and doubling time were calculated according to the Eq. (1) and Eq.(2), respectively.

$$\mu = \frac{\ln Xt - \ln X_0}{t} \quad (1)$$

$$dt = \frac{\ln 2}{\mu} \quad (2)$$

Where X_0 denotes the initial biomass concentration after the elapsed time t and Xt is the biomass concentration after the elapsed time [9].

2.7. Analytical method

Lignocellulosic contents of Napier grass was analyzed according to the Chesson-Datta method [10]. SSF products (bioethanol, acecate, glucose and xylose) were analyzed using high performance liquid chromatography (HPLC) equipped with an ion moderated partition chromatography column, Aminex HPX-87H (Bio-Rad, CA). The HPLC was used in combination with a Waters (2414) refractive index detector. The flow rate of the mobile phase (5 mM of H_2SO_4) was adjusted at 0.6 ml/min and the temperature was set at 60 °C. The amount of reducing sugar released by saccharification and remained in the supernatant was determined by Dinitrosalicylic Acid (DNS) method [11]. Protein concentration was measured by the method of Bradford [12] using bovine serum albumin as a standard. Filter paper activity (FPA) was determined as described by Ghose [13]. The enzyme activity was expressed in international units (IU), defined as the amount of enzyme required producing 1 mmol of glucose per minute under the assay condition [14].

3. Result and discussion

3.1. Alkaline pretreatment for Napier grass

In this study, the lignocellulosic component of Napier grass before and after pretreatment with 1.5% (w/v) NaOH solution were determined (Figure 1). After the pretreatment process, lignin and hemicellulose content in Napier grass were decreased, however cellulose content was increased. The percentage of lignin removal in this study was 54.9%. It is clear that alkaline pretreatment aims to eliminate lignin to increase porosity and specific surface area and lead to an increase in the enzymatic digestibility of biomass. The extractive content of Napier grass was about 32.5% and increased to 38.63% after the pretreatment process. It means lignin removal have made the extractive is more accessible to the enzyme or microorganism. Cellulose content was also increased from 30.7% to 37% after the pretreatment process. This percentage of levitation occurs based on solvation and saphonication by alkaline catalysts, which induce depolymerization and division of lignin-carbohydrate linkages, resulting the substance that are not biodegradable are more accessible by extracellular enzyme [15]. In addition, these chemicals are able to break the ester bonds between lignin, hemicellulose and cellulose so that lignin and hemicellulose are concurrently removed [16].

3.2. Growth kinetics and substrate consumption of Neurospora sitophila

The growth of fungi in submerged fermentation can be determined by estimating the biomass dry weight concentration. In general, four phases in the growth of filamentous fungi have shown, namely, the lag phase, the log phase, the stationary phase, and the death phase. Figure 2 depicts the growth curve of fungi during cultivation with Napier grass. However, the fungal growth has increased significantly within 24 h of incubation thus the lag phase of *N.sitophila* can not be distinguished. According to Dewi [17], the adaptation or lag phase of fungi should be found at 12-16 hours of cultivation. After that, fungal growth reached

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the log phase in 16-24 h of cultivation and leaded to increasing biomass exponentially. Table 2 summarises the specific growth rate and doubling time of *N.sitophila* on various Napier grass substrate concentrations. In this study, specific growth rate of *N.sitophila* in 10 g l^{-1} of substrate concentration was 0.136 h^{-1}. Similar result was reported by Oguntimein [18] that obtained the specific growth rate of *Neurospora* sp on corn stover 1% (10 g l^{-1}) was about 0.14 h^{-1}. Thereafter, the biomass of fungal was decreased on 48 h of cultivation onwards. This phenomenon occurs because the availability of lignocellulose as a carbon source is depleted with increasing cultivation time. In addition, Figure 5 also shows reducing sugars in the medium was also decreased. This result shows nutrient depletion was attributed to growth restriction. This result was similar to other study [19] that showed biomass of fungal *Neurospora* sp decreased in three days of cultivation.

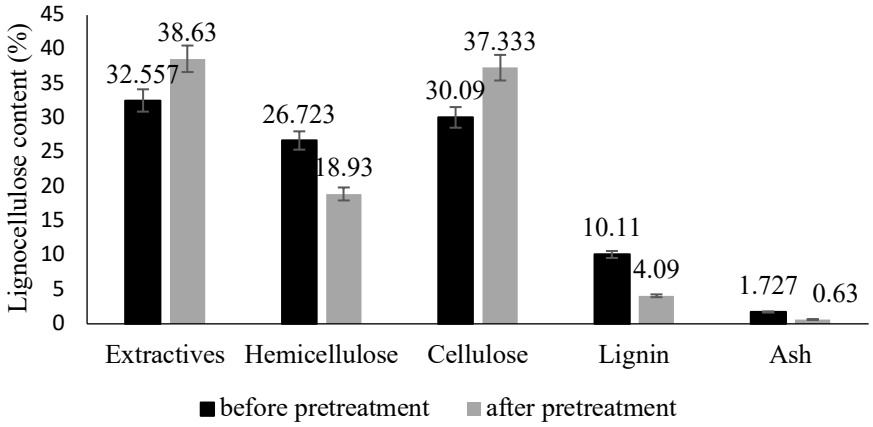

Figure 1 Composition of lignocellulose on Napier grass after pretreatment*

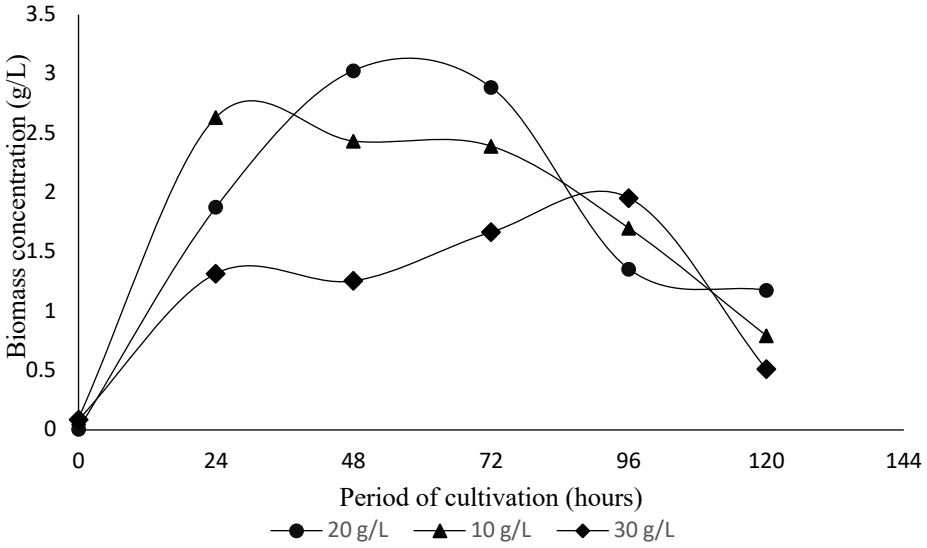

Figure 2 Growth curve of *N.sitophila* during simultaneous saccharification and fermentation in various substrate concentration of Napier grass

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N.sitophila grown best in 20 g l^{-1} of Napier grass as indicated by the highest rate of specific growth and the shortest hour of doubling time. Growth of *N.sitophila* on the substrate of 20 g l^{-1} was performed better than substrate concentration of 10 g l^{-1}. The increasing of initial substrate concentration induced cells to grow faster so the biomass concentration will also increase. However, *N.sitophila* on the substrate of 30 g l^{-1} having a slow growth rate compared to 20 g l^{-1} substrate concentration. This may be occured due to low oxygen transfer to fungi cells that are close contact with the substrate. As shown in Figure 3.a, the cell did not grow well since the presence of substrate restricted the oxygen transfer. Oxygen is the elementary components for substrate utilization. Hence, the limited supply of oxygen to a cell will lead to a slow growth rate [20].

Profile substrate component availability was reported in Figure 4. Based on the graph., it showed at 24 hours of cultivation, *N. sitophila* consumed simple sugars in extractives and at same time, it started to degrade the cellulose and hemicellulose by secreting hydrolytic enzyme [21]. As a consequence, after 24 hours, for the treatment using 10 g/L of Napier grass in the culture showed in a low content of sugar sources (extractives, hemicellulose and cellulose) hence the growth of cell also decreased. A different result obtained from the treatment using 20 g l^{-1} of Napier grass which was shown a high content of sugars and started to decreased gradually. For the treatment using 30 g l^{-1}, the substrate availability was gradually decreased and thereafter became stationer since the substrate components were depleted. However, there an error was detected at 72 hours of cultivation which shown an increase of cellulose content whereas the content of oteher components were decreased.

3.3. *Production of crude cellulase extract*

Profile of cellulase activity on various the Napier grass substrate concentrations were presented in Figure 5. Cellulase enzyme activity has been detected in 24 h of cultivation. Based on statistical analysis, there was not any significant difference ($p>0.05$) of enzyme activity recorded as varying the substrate concentrations. However, the enzyme activity at 20 g l-1 of Napier grass is greater than 10 g l-1 of substrate concentration. But, the enzyme activity was relatively declined on the culture with substrate concentration of 30 g l-1. Cheng and Anderson reported that substrate concentration is one of the main factors that affect the yield and initial rate of enzymatic hydrolysis of cellulose. At low substrate levels, an increase of substrate concentration normally results increases the yield and reaction rate of the hydrolysis [22]. However, this result showed that the enzyme activity at 30 g l-1 is lower than 20 g l-1

The fungal growth could be correlated with enzyme activity. This is might correlated with poor fungal growth which was observed on treatment with 30 g l-1 substrate concentration Napier grass in the culture. It seemed that the substrate was too compact lead to reduce surface contact area [21].

The highest enzyme activity on 10 g l-1 substrate concentration was 0.185 FPU/ml with 48 h of cultivation (Figure 6.a). Similarly, Oguntimein [18] also stated that the activity of cellulase enzyme produced by Neurospora on 1% corn stover and Chinese baggase was 0.15-0.18 FPU/ml. This activity also occurred in 40-60 hr of cultivation. The enzyme activity became stationer after 48-72 hours of cultivation. After 72 hours of cultivation, cellulose availability is low hence enzyme activity also was decreased. The growth of biomass also decreased and could cause factors in enzyme activity to decreased. A different result was obtained from 20 g l-1 substrate concentration. Figure 6.b shows the activity of cellulase has a stationary trend then peaked at the end of cultivation (120 hrs). When the sugar is present in high concentrations, it could inhibit cellulase performance. After the reducing sugars is started to depleted, the fungi will degrade hemicellulose and cellulose thus the availability of cellulose and hemicelluose in substrate was decreased (Figure 4). Generally, cellulase produced from substrate concentration of 20 g/L has greater stability than other treatments. At the end of cultivation, the cellulase activity remains high, approximately 0.28 FPU/ml. By increasing enzyme stability, the yield of products could be increased [23].

There was no salient increase of enzyme activity at 30 g l-1 substrate concentration even though the substrate concentration was the highest. This condition occurred because the fungi did not growth well on substrate concentration of 30 g l-1 compared to other treatments. When growth is not optimal, it will influence the performances of enzymes. Cellulase activity was reached its peak when 72 hours of cultivation thereafter decreased in the stationary phase of fungal growth. Enzyme activity was low also because the sugar concentration is high and may lead to repression of cellulase expression [24].

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(a) (b) (c)

Figure 3 Growth of *N.sitophila* in various Napier grass substrate concentrations (a) 10 g l^{-1} (b) 20 g l^{-1} (c) 30 g l^{-1}

Table 1 Specific growth rate and doubling time of *N. sitophila* in various Napier grass substrate concentrations

Treatments	Specific growth rate ($hours^{-1}$)	Doubling time (hours)
10 g l^{-1}	0.1363	5.0845
20 g l^{-1}	0.2274	3.0478
30 g l^{-1}	0.1107	6.2609

3.4. Ethanol Production

Neurospora sitophila is one of the few microorganisms that able to convert cellulose and hemicellulose into ethanol in two stages, by producing the required hydrolytic enzymes during aerobic growth stage and fermenting the produced simple sugars to ethanol in anaerobic or micro-aerobic conditions (fermentation stage). Ethanol was produced based on the Embden-Meyerhof glycolytic pathway with resulting 2 mol of ethanol being produced per a mol of glucose utilized [25]. Neurospora sp secreted alcohol dehydrogenase (ADH) which is an enzyme for ethanol production. Profile of bioethanol production on various Napier grass substrate concentrations was investigated in this study (Figure 7). The results of ethanol, reducing sugars, acetate concentration and ethanol yield from all treatments were presented in Table 2. These results indicate an increasing substrate concentrations will lead to an increasing concentration of ethanol that produced. Based on this study, it was found that ethanol was already produced in 24 hrs of cultivation in the fermentation of 10 g l-1 substrate concentration. It indicates that the ADH enzyme has performed to produce ethanol. However, also in this treatment, the ethanol concentration was decreased with increasing time cultivation. It seems the cells started to grow, the sugar availability was starting depleted to low concentration of 0.07 g l-1 (Table 2). The low concentration of sugars were not sufficient for fungi, and as a result, Neurospora sp utilized the ethanol produced as a carbon source for growth. Recent studies by Lin [26] and Dewi [17] also observed that under some conditions, Neurospora sp can utilize and metabolize the produced ethanol for growth. Alcohol dehydrogenase 1 gene (adh-1) is reported to be responsible for ethanol degradation while the adh-3 enzyme is responsible for ethanol synthesis [27].

Ethanol produced by Neurospora sp on substrate concentration of 20 g l-1 in 48 h of cultivation was 0.189 g l-1. There was an increase in ethanol concentration as time cultivation increased because reducing sugar concentrations were remained in high value. This happens during the process of lignocellulose degradation leads to sugar availability to produce metabolites such as ethanol. Whereas, the highest ethanol was achieved on substrate concentration of 30 g l-1. It reached about 1.25 g l-1 of ethanol at 96 hours of cultivation. However, there was a decline in ethanol concentration at 120 hrs of cultivation. This decrease might be caused by the formation of acetate, resulting in lower yield of ethanol. A similar result was obtained from the other study [17] which indicates that the formation of acetate was a simultaneous phenomena with ethanol reduction.

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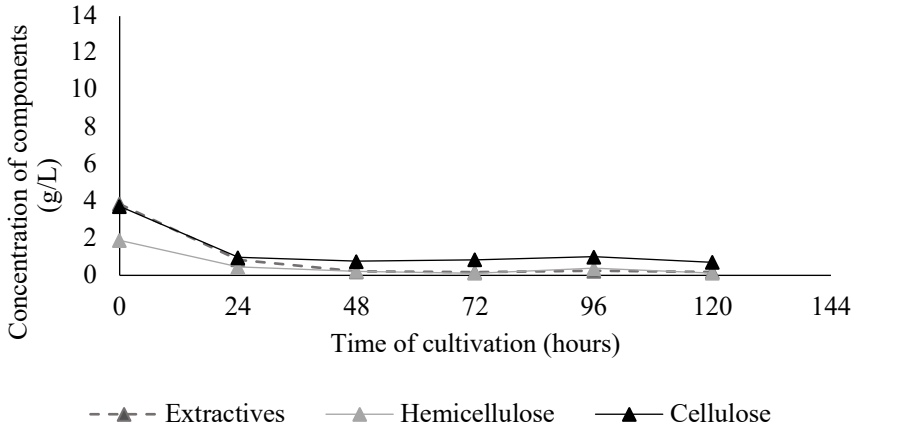

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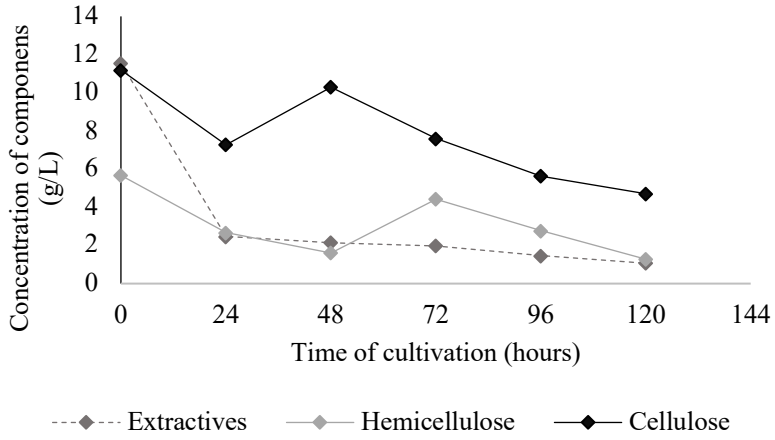

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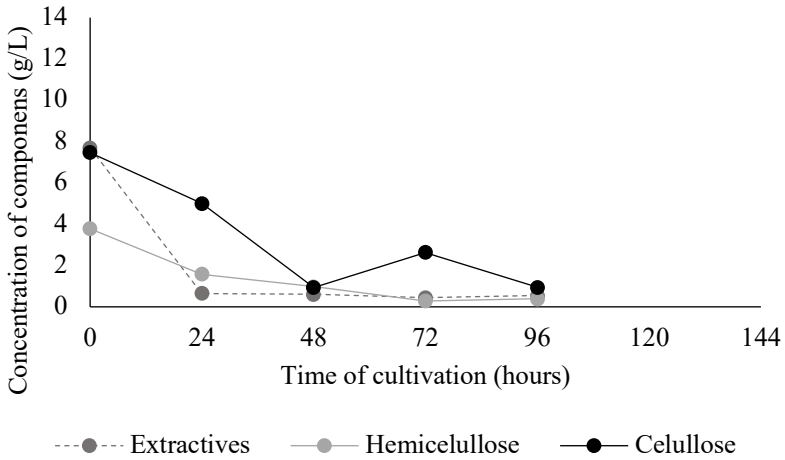

(c)

Figure 4 Lignocellulosic components of Napier grass after simultaneous saccharification and fermentation a) 10 g l^{-1} b) 20 g l^{-1} c) 30 g l^{-1}

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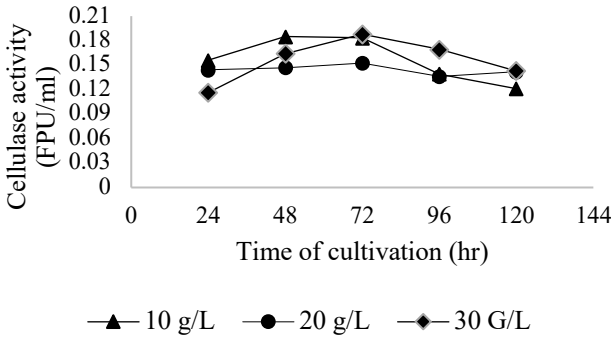
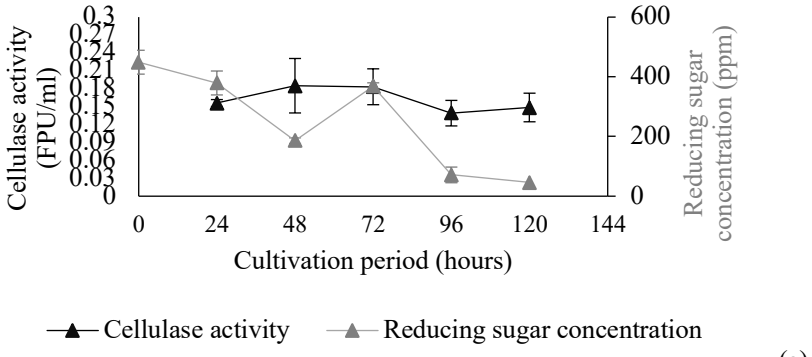


Figure 5 Profile cellulase activity in various Napier grass substrates concentration



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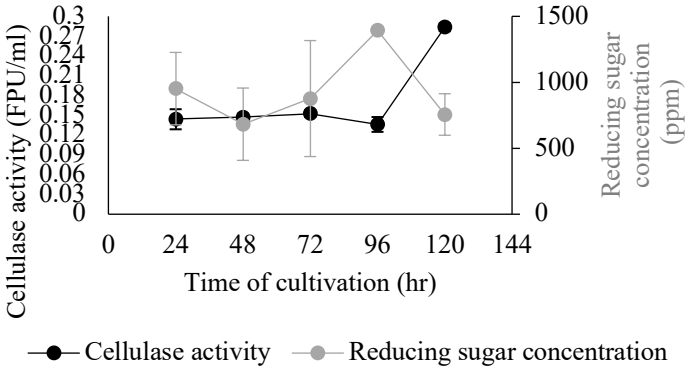

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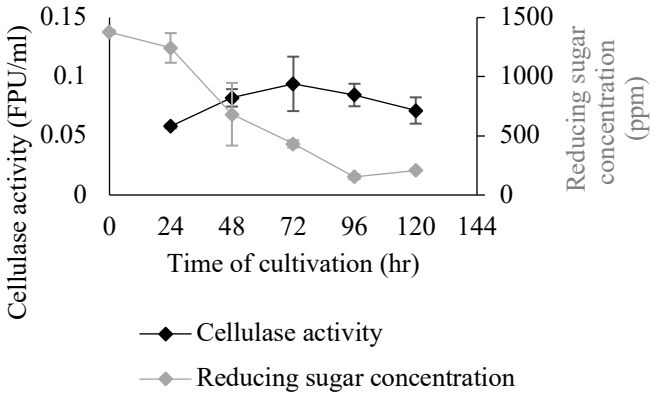

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Figure 6 Profile cellulase activity and reducing sugar concentration from simultaneous saccharificaton and fermentation using various Napier grass substrate concentration a) 10 g l^{-1} b) 20 g l^{-1} c)30 g l^{-1}

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Table 2 Reducing sugars concentration, ethanol concentration, ethanol yield and acetate concentration in various Napier grass substrate concentrations and time of cultivation

Substrate concentrations	Cultivation period (hrs)	Reducing sugars concentration (g l^{-1})	Ethanol concentrations (g l^{-1})	Ethanol yield (g ethanol/g cellulose)	Acetate concentrations (g l^{-1})
10 g l^{-1}	24	0.38	0.42	0.15	0
	48	0.17	0.17	0.06	0
	72	0.40	0.29	0.10	0
	96	0.07	0.19	0.07	0
	144	0.05	0.21	0.07	0
20 g l^{-1}	24	0.95	0	0	0.23
	48	0.68	0.19	0.02	0.01
	72	0.88	0.73	0.09	0.84
	96	1.39	0.53	0.08	0.06
	144	0.76	0.85	0.12	0.09
30 g l^{-1}	24	1.24	0	0	0.16
	48	0.68	0.99	0.23	0
	72	0.43	0	0	0
	96	0.15	1.25	0.30	0
	144	0.20	1.19	0.22	0.02

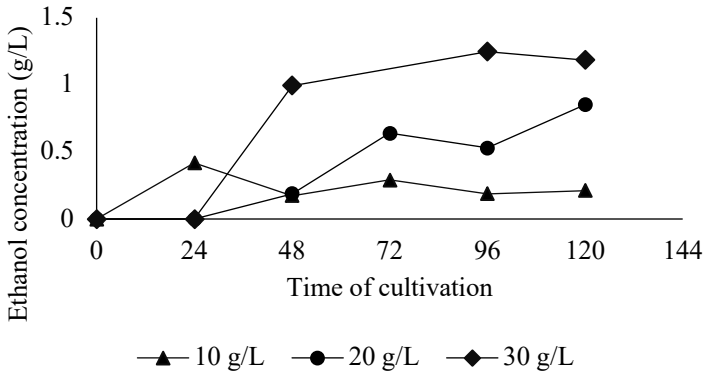

Figure 7 Profile of ethanol accumulation in various Napier grass substrate concentrations

This study showed that ethanol accumulation was maximum at the stationary phase. This result also was reported by Colvin [26]. Ethanol produced in this study still has a low value in comparison to other studies. Colvin reported that accumulation ethanol by Neurospora sp can reach 4.5 g/L. Dewi also observed that the highest ethanol produced using Neurospora sp on leaves of vetiver grass was 4.5 g/L within 48 h of cultivation. This is could be correlated that the culture was still containing oxygen. The headspace still containing 60% of void space since the working volume is 40% of total volume. This indicates the bioreactor headspace is occupied by air and could enhance enhancing oxygen mass transfer to a culture. Eakin, R. T., & Mitchell reported a wild type strain of Neurospora has consumed oxygen at rate of 66 mmol of oxygen/minutes [28]. Based on theoretical calculation, during the cultivation time, the oxygen in the culture remain high and have not reach microaerobic condition. As a result, the fermentaion process was inhbited. This phenomenon is probably related to Pasteur effect. The Pasteur effect, the inhibition of fermentation by respiration, may be operative in Neurospora sp [25].

The ethanol yield from all treatments are shown in Table 2. Based on the result, the highest ethanol yield was 0.30 g/g cellulose conducted by 96 hours of fermentation. This value was lower than the study of Ingram and Doran [29], that reported ethanol yield could reach 0.30 g/g cellulose based on lignocellulose material. Low ethanol yield indicates the

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substrate that consumed is not fully used for ethanol production. Substrate utilization for cell maintenance was not led to biomass formation [9]. Fermentation products and substrate analyzed in HPLC and the chromatograph had shown in Figure 8.

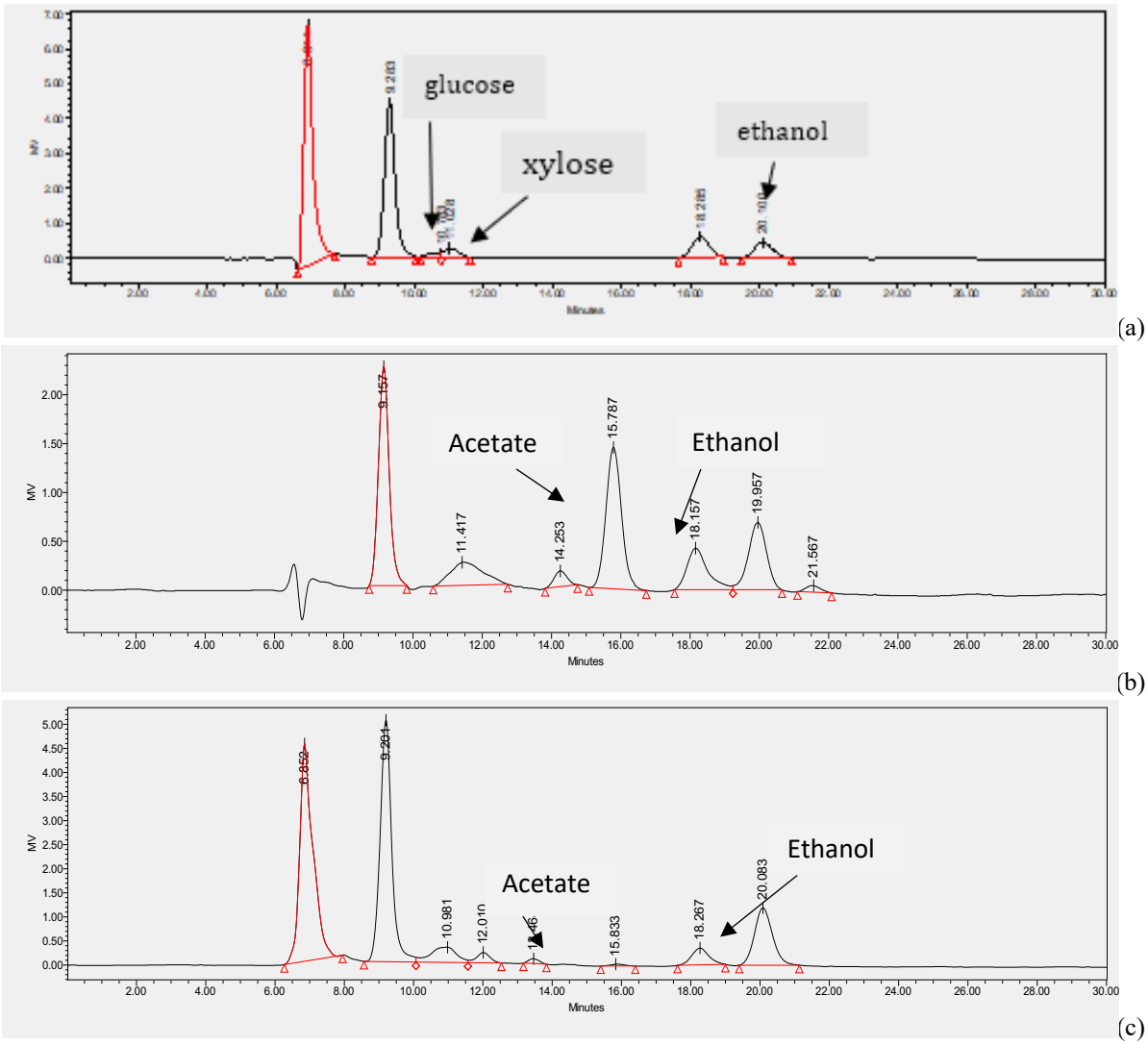

Figure 8 Chromatograph of fermentation samples using various substrate concentrations of Napier grass a) 10 g l^{-1} b) 20 g l^{-1} c) 30 g l^{-1}

However, there is a room for further improvement by optimizing the microaerobic condition for bioethanol production. In addition, it is also possible for using Neurospora sp and yeast simultaneously for enzyme production and obtaining bioethanol production.

At T_1 and T_2, the diversity index value in the treatment does not have a certain trend. Spraying herbicide may not have a significant effect on the species diversity index at the time of T_2. But on T_3 (before harvest), the diversity index value in all treatment groups experienced an increase (Figure 4).

4. Conclusion

The highest cellulase enzyme activity produced by *N. sitophila* is 0.28 FPU/ml occurred at 120 hours of cultivation and 20 g l^{-1} substrate concentration. However, the ethanol yield was 0.30 g/g cellulose, which achieved at 30 g l^{-1} substrate concentration and by 96 hours of fermentation. Napier grass could serve as a good substrate for production of value-added products such as crude extract cellulase enzyme and bioethanol through simultaneous saccharification and fermentation using *N. sitophila*.

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An Increase of Silicon Recovery from *Oryza sativa* L. Husk by Cow Rumen Fluid Treatment

Muhammad Rizki Fajar Ilhamy , Khairul Hadi Burhan, Robert Manurung*

School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

Corresponding author; e-mail: manurung@sith.itb.ac.id
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Abstract

Si is one of the beneficial nutrients and has various essential roles to cope with stresses situation in the graminaceous plant. The availability of Si on earth is abundant however the form that can be readily uptake by the plant is limited, and without external addition, the silica content in the cultivated land would be depleted and decreasing plant growth, recycle Si from the decaying plant is a better alternative to protect from that situation. Rice husk is a leftover paddy by-product having significant Si content that can be reused. This study offered a biological way to treat the rice husk using rumen fluid as a better alternative to the high energy consuming thermal procedure. Rice husk was macerated in rumen fluid, the kinetic and model of lignocellulose degradation were evaluated. The study in Si release in liquid media i.e. 0.05 M hydrochloric acid, 0.1 citric acid and aquades, and Si extraction in alkaline solution 1 M KOH using the rumen-treated husk showed that this method could be a better alternative to develop high Si content of solid and liquid fertilizer, respectively.

Keywords: biodegradation, lignocellulose, rice husk, rumen fluid, silica

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1. Introduction

Silicon (Si) has various essential roles in graminaceous plants i.e. wheat, paddy, corn, etc. Its existence is found to relieve several forms of biotic and abiotic stresses. Studies reported that Si provides a mechanical barrier through the deposition in non-soft tissue as protection from insects and pathogens, and as a chemical inducer i.e. alters the presence level of herbivore-induced plant volatiles compound [1]. Moreover, Si application is found to alleviate the stresses caused by temperatures, salinity, and elements toxicity [2]. It increases the tolerance of salt concentration, protects from the oxidative stress damage, and reduce the inhibitory effect of Na^+ [3]. The silicon soil amendment succeeds to increase the yield and supresses the diseases in paddy due to the thickening in the epidermal layer to reduce palatability and digestibility in the pests and stimulation of insects-resistance biochemical pathway [4].

The availability of Si is abundant in the world. It is the second most abundant element in the earth's crust in crystalline form, quartz, or silicon oxide (SiO_4, SiO_2). However, not all of the available Si can be taken directly by plants, the crystal phase is un-dissociated due to having lower solubility which is 10^{-4} M than the amorphous one, $10^{-2.74}$ M that is normally present in soil [5]. The absorbed Si is distributed and accumulated in the aboveground plant biomass, particularly in straw and grain tissue and having strong correlation with the presence of other elements i.e. C, N and P [6]. The Si-derived from plants is very essential as the accumulated Si, in the form of phytolith, will be recycled in the soil solution and can be reabsorbed by the new plants once the decay of plants occurred [7].

Rice (*Oryza sativa* L.) is commonly known as Si accumulator, more than 50% of Si uptake is deposited in straw, which is 59% of the total biomass [8]. While the rice husk contains Si as much as 91.42% of its ash content, and it could achieve up to 20% of rice husk in dry weight, and the rest comprise cellulose (35-40%), hemicellulose (15-20%), and lignin (20-24%) [9]–[11]. The common method in Si recovery from rice husk is through chemical and combustion process, the product i.e. husk charcoal or biochar is subjected to alkaline reaction and then apply acid solution to precipitate Si in a form of H_2SiO_3 [12]. These robust methods have disadvantages such as high-energy usage due to high-temperature operation, and requires neutralization step due to strong alkaline and acid consumption in extraction processes [13].

A biological approach in rice husk treatment is a potential alternative in Si recovery, as such, it can be celebrated in mild temperature and consuming less toxic chemicals. The process proposed was conducted using rumen cow fluid due to the composition consists of lignocellulose-degrading bacteria and enzyme mixture [14]. Studies reported that rumen fluid has the activity of each complex enzyme cellulase, hemicellulase, and lignolytic enzyme with the highest value at 80, 250, and 35 U/L respectively [15]. Therefore, the lignocellulose structure of rice husks can be loosened enabling the access of Si-leaching chemicals. The investigation of rumen fluid application for this purpose was rarely studied, commonly it applies to lignocellulose substrate for methane formation and nutritional uptake for animal feed. Using cattle rumen fluid for riverbank grass waste treatment at 38.6°C resulted in 50% decrease of chemical oxygen demand which was representing the amount of organic compound in the mixture [16]. Pretreatment of rice straw, to produce biomethane, using rumen fluid succeeded to reduce the total solid up to 65% after 5 incubation days [17], and when applied to the grass clipping to enhance hydrolysis performance, the content of soluble chemical oxygen demand increased up to 6.5 g/L after 3 days of incubation period, indicating the lignocellulose hydrolysis occurred [18].

The purpose of the study was to examine the influence of incubation time in lignocellulose degradation using rumen fluid on the amount of Si extracted using maceration method and to evaluate the kinetic model in rice husk lignocellulose biodegradation. The objective was to increase the Si release in several liquid media with the pretreatment using rumen fluid based on incubation time and to compare the extraction results between biodegradation and thermos-degradation treatment, and to determine each kinetic coefficient in cellulose, hemicellulose and lignin degradation. The information given in this investigation can be used to develop Si-containing solid and liquid fertilizer with more efficient in energy and chemicals consumption.

2. Materials and Methods

2.1. Materials

The rice husk was collected from Bunihayi Village, in Subang, West Java, Indonesia. It was air-dried at room temperature and ground to the size of 40-mesh using a kitchen grinder (Philips, HR2116). The rumen fluid was obtained from a local slaughterhouse in Cikuda village, in Jatinangor, West Java. The fluid was kept in the temperature range of 38-40°C close to the body temperature of the ruminants [17]. The chemicals used such as H_2SO_4, KOH, 1-amino-2-naphthol-4-sulphonic acid, molybdic acid, hydrochloride acid, oxalic acid, and acetic acid were analytical grades and obtained from chemicals warehouse of School of Life Sciences and Technology Institut Teknologi Bandung.

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2.2. Methods

The experiments focused on husk lignocellulose degradation in variation of time, and the rumen-treated result was compared to the husk char with a certain amount of carbon content. The result from optimum time would be subjected to Si release assay in liquid media, and the comparison of Si extraction using alkaline solution for husk charcoal, rumen-treated husk and untreated husk as a control. This experiment was conducted in duplicate.

2.2.1.Husk char synthesis

The rice husk of 10 g in a lid covered chamber was placed in the furnace. It was maintained at various temperature 350ºC for 90 minutes [19]. The ash content of the char resulted will be determined at the temperature of 950ºC for 60 min [20].

2.2.2.Husk degradation using rumen fluid

The rice husk powder of 10 g with 15% moisture content was added into 200 ml rumen fluid in a 300 ml Erlenmeyer flask. After that, it was incubated at 38ºC, for 24, 48, 72, 96, and 120 hr in an incubator shaker. The mixture then was filtered through Whatman No.1 filter paper and dried at 105ºC for 24 h.

2.2.3.Lignocellulose content measurement

Lignocellulose content in each sample was measured using Chesson-Datta method [21]. The sample of 1 g was added into 150 ml distilled water and refluxed for 2 hr at 100ºC. After that, it was filtered, and dried under 105ºC for 24 hr to determine the hot water soluble (HWS) content. The previous treatment was subjected to hemicellulose determination, 0.5 M H_2SO_4 of 150 ml was added, and refluxed at 100ºC for 2 hr. Then it was filtered and dried as the previous one. The hemicellulose-lack residue was added into 10 ml of 72% (v/v) H_2SO_4 and incubated in room temperature for 4 hr, after that it was diluted to 0.5 M, and refluxed for 2 hr at 100ºC. After filtered and dried, the cellulose content was measured. The residue left was then pyrolised at 575ºC for 2 hr. After that, the sample was cooled, and the ash and lignin content were determined. The entire component was determined using the equation as follow:

$$\text{Hot water soluble}\left(\text{HWS}\right)\left(\%\right)=\frac{a-b}{a}\times 100\% \quad [1]$$

$$\text{Hemicellulose}\ \left(\%\right)=\frac{b-c}{a}\times 100\% \quad [2]$$

$$\text{Cellulose}\left(\%\right)=\frac{c-d}{a}\times 100\% \quad [3]$$

$$\text{Lignin}\left(\%\right)=\frac{d-e}{a}\times 100\% \quad [4]$$

$$\text{Ash}\left(\%\right)=\frac{e}{a}\times 100\% \quad [5]$$

Where a, b, c, d, and e were the initial sample mass, mass without HWS, mass without HWS and hemicellulose, mass without HWS-hemicellulose-cellulose, and ash, respectively.

2.2.4.Degradation efficiency and Kinetics

The degradation efficiency of the lignocellulose content of the husk was calculated according to Eq. 6 [17] as follows:

$$\eta_j=\frac{m_{i,ini}-m_{i,fin}}{m_{i,ini}} \quad [6]$$

Whereas the kinetics mechanism was evaluated using the Michaelis-Menten equation as a form below:

$$\frac{d\eta_i}{dt}=v_{max,i}\frac{\left(1-\eta_l\right)}{K_{m,i}+c_{o,i}\left(1-\eta\right)} \quad [7]$$

Where η_i is degradation efficiency [dimensionless] of composition i such as cellulose, hemicellulose, or lignin of the rice husk, v_{max} is the maximum rate of degradation [g/L-h], Km is half-saturation constant for each component concentration [g/L], and $c_{o,i}$ is the initial component concentration [g/L].

2.2.5.Si release assessment from rumen-treated husk and husk char in liquid medium

The liquid media used were distilled water, 0.1 M citric acid solution [22], 0.05 M hydrochloric acid [23], and at this step the focus was emphasized to performance of each type sample in Si release. A total of gram from each sample was added into 200 ml of each medium and incubated at room temperature in an orbital shaker at 130 rpm. The observation was carried out at a specified time for 36 hr. The silica content in the liquid media was analysed using the ASTM Method: Standard Test Method of Silica in Water (2000) [24].

2.2.6.Si extraction from rumen-treated husk and husk char

A total of 25-gram amount from each sample was added to 100 ml of 1 M KOH solution. It was incubated at 38ºC, 225 rpm, and the observation was taken in 2 hr interval time. The mixture was filtered through a Whatman 1 filter paper and the silica content in the filtrate was measured according to the previous method.

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3. Results and discussion

3.1. Husk Char and Lignocellulose degradation of rumen-treated husk

Thermal process resulted in husk char with carbon content (volatile matter and fixed carbon) as much as 72.38 %, this value was about the same with several reports conducting the procedure at the same temperature which was 72.8% on dry basis [25], [26]. Whereas the initial content of cellulose, hemicellulose, and lignin in rice husk for this investigation were 43, 17, and 26% by weight, respectively, the value obtained in this investigation were not the same to other literatures with the average such as 33.43, 20.99, and 18.25% respectively [27], [28]. The treatment of husk by using rumen resulted in an increase of the degradation with the time courses as shown in Fig. 1. The highest efficiency achieved for cellulose, hemicellulose, and lignin were 89, 17, and 21% respectively after a 120-hour period of incubation.

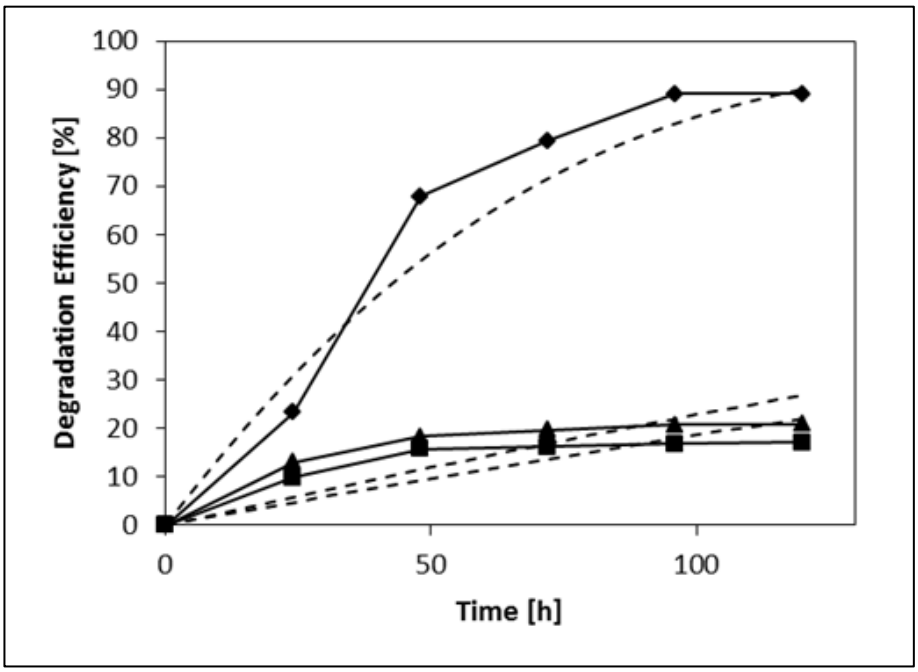

Figure 1 Lignocellulose profile of rumen-treated husk for 120 hours (♦) cellulose, (■) hemicellulose, and (▲) lignin content, the dashed lines are the predicted model kinetics.

The cellulose degradation was the highest, and the significant increase took place from 24 to 96 h time courses, after that it tended to be stationer, while the hemicellulose and lignin showed the similar pattern. Treatment using rumen fluid on rice biomass found to be effective in reducing lignocellulose as investigated in rice straw treatment that the stationer level was achieved at 96 h of incubation time with the efficiency for cellulose, hemicellulose, and lignin was 46, 59, and 21 % respectively [17], comparing with this results the current investigation had higher activity in cellulose degradation and lower for hemicellulose one.

This results also implied that the rumen fluid had specialized enzymes secreted by the indigenous microorganisms for lignocellulose degradation, the presence of this microbial community have been detected through several modern molecular techniques [29]. Several investigations in the potential microorganism of rumen fluid were carried out, a significant reduction in content was achieved on cellulose in 72 h treatment as much as 23%, and it was found that the dominated microorganism was *Firmicutes*, and this particular group of bacteria are the potential cellulase producer [30], [31]. Another investigation also showed that the particular microorganism has the ability to degrade lignin due to extracellular peroxidase [32]. The lignin degradation in this investigation was higher than the hemicellulose. Its value was almost close to other investigations when using rumen to degrade wheat straw and corncob, with efficiency at 25.5 and 30% respectively [33].

3.2. Kinetic model of husk lignocellulose degradation

The calculated kinetics parameter v_{max} for cellulose, hemicellulose, and lignin can be shown in the Table 1.

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Table 1 Kinetic parameters value i.e. maximum rate (v_{max}), and saturation constant (K_m) for lignocellulolytic activity in the rumen fluid.

Component	Kinetic parameters	
	v_{max} (g/L-h)	K_m (g/L)
Cellulose	0.77	33.11
Hemicellulose	0.04	13.25
Lignin	0.08	19.87

The kinetics for lignocellulose degradation using rice husk as a substrate particularly by a rumen fluid is not yet reported, the kinetic coefficients calculated were the apparent values not the intrinsic one due to the biocatalyst consists of many microbes. However, as a comparison the values could be compared to the intrinsic values of kinetic for cellulase produced by *A.fumigatus* Z5 at 37.8 g/L for K_m, and 437.3 µmol/min-mg for v_{max} [34], and for lignolytic enzyme from *Pleuorotus* sp. was calculated to be 250 mM and 0.33 µM/min for K_m and v_{max} respectively [35].

3.3. *Silica release from char and rumen-treated husk*

At this step, the study was trying to mimic the natural environment of soil for Si leaching from organic material using both weak and strong acids, these acid concentrations were selected due to commonly applied in inorganic material leaching in the soil [22], [23]. Based on the solvent, all type of sample had the highest solubility in 0.05 M hydrochloric acid solution, whereas the lowest for each sample category was obtained in distilled water solvent (Figure 2.). For the across type sample rumen-treated husk in HCl solution had the most soluble Si, after achieved the equilibrium condition at 24 hr, after that it was followed by husk char in HCl, then rumen-treated in 0.1 citric acid solution, then husk char in citric acid and control in the HCl. The less soluble Si came from control and husk char in distillate water.

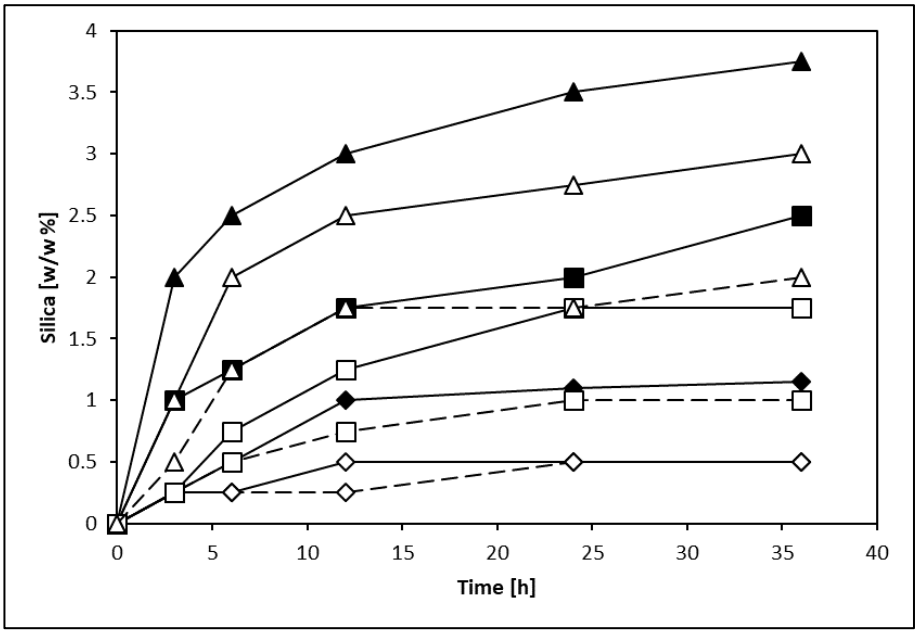

Figure 2 The dissolved silica in sample weight percentage of each treatment i.e. rumen treated husk (full mark), husk char (empty mark) and control (empty mark + dashed line) in several media i.e. distilled water (diamond), 0.1 M citric acid solution (square), and 0.05 M HCl solution (triangle)

The maximum values achieved were 1.15, 2.5, and 3.75% weight of the treated husk in the distilled water, 0.1 M citric acid, and 0.05 M HCl solution respectively. The solubility from all conditions were still far from the total content of silica in the husk at 17%. Whereas for the husk char, the highest yield percentages of silica were 3.0, 1.75, and 0.5%

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for hydrochloric acid, citric acid, and distilled water, respectively. For the control (untreated husk), the highest value was 2.0, 1.0, and 0.5% by sample weight for the corresponding medium, hydrochloric acid, citric acid, and distilled water respectively.

Comparing to the distillate water as the control solvent, the significant increase of dissolve silica occurred in acid solutions, and it was obvious that the type of acids had more influence than the amount of acids concentration as the molarity applied for strong acid, was less than weak acid. The strong acid leads to deconstruct the organic matter due to high affinity of nucleophile attack on the biomass material hence the fibrous structure was loosened, it leads to it wide application in biomass acid hydrolysis [36], as organic material, the decomposed rice husk will allow more inorganic material, which is dominated by Si, to escape. An investigation strong acids including hydrochloric higher than 2.0 M in the treatment to extract silica from biomaterial including rice husk found that it increased organic matter removal impurities [37] and also increased the specific surface area of the particles [38]. Because of classified as dangerous chemicals, strong acid was not applicative, the organic acid as known as weak acids would be highly recommended particularly the citric acid, one of the majority acids in the soil [39].

The extracted silica from the husk char was lower than the yield obtained in the rumen-treated husk. This occurred because the content of extractable organic carbon represented by the lower content of carbon content, and an investigation was shown that the presence of charcoal in the soil lowered the content of extractable organic carbon[40]. The less dissolved char led to less silica released. It preferred to act as an adsorbent rather than soluble compound in the liquid medium [41].

As the previous explanation, the higher acid concentration led to organic matter leaching [37]. However, in this experiment the acid concentration applied was lower than 0.5 M. As such, it released less silica compare to the treated husk. The untreated rice husk is an analogue to its utilisation directly without any preliminary procedure, and it would be ineffective because of the longer time required in degradation. It had been studied that mushroom, *Pleurotus ostreatus*, required 35 days to increase rice husk digestibility up to 79.4% [42].

3.4. Si extraction using alkaline solution

This step was trying to figure out the potential of biological-treated husk as a substitute process to the thermal treatment in a set of Si extraction. Commonly, the Si extraction directly from the char using alkaline solution was performed to obtain Si solution and highly porous bio-char [43]. The extraction effectiveness in alkaline solution was observed for each husk treatment. The husk char content released the highest silica at 25% of the ash weight. And then followed by rumen-treated husk, and untreated (control) husk as much as 17, and 7%, respectively as can be shown in Figure 3.

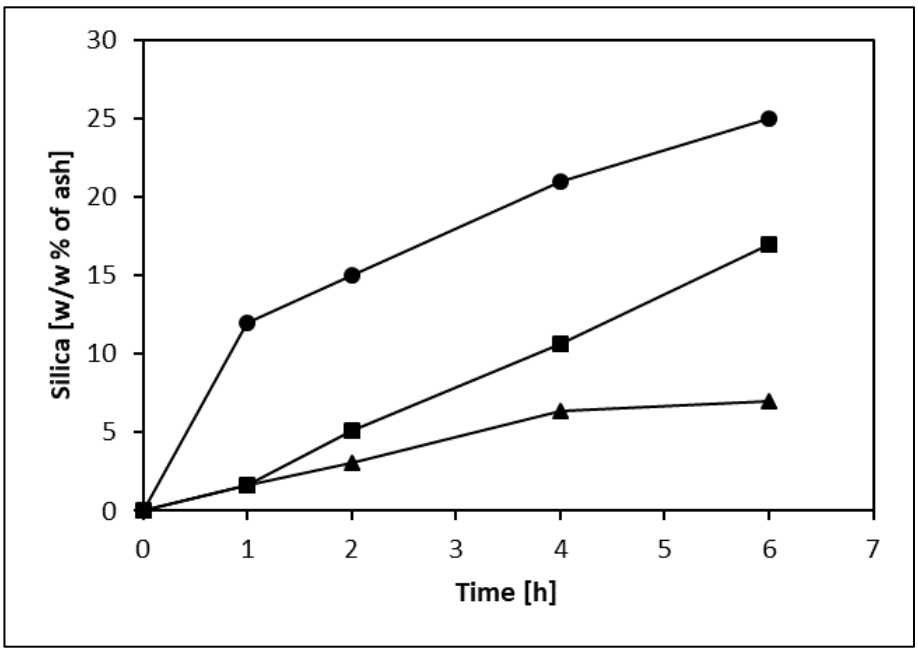

Figure 3 The dissolved silica in weight percentage of the ash from the each husk; (▲) the untreated husk or control, (■) the rumen-treated husk, (●) the char husk, in 1 M KOH solution

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Extraction using the alkaline solution resulted in silica driven off from the char and ash. char with 1.0 M NaOH solution treatment had a more porous structure than untreated based on SEM results [43]. Moreover, 85% silica from ash was recovered after 1.0 M NaOH treatment for 1 hr followed by the addition of 1.0 M hydrochloric acid to attain pH at 7 [44]. França et al. investigated silica extraction from rice husk ash using KOH solution with various concentration and showed that 32.3% recovery from ash was achieved using 1.0 M solution for 6 hr. [45]. The difference between biological treatment and thermal treatment was not too far or less than 50%, it means that biological process could be an alternative to the thermal one in a series of extraction procedures.

4. Conclusion

The rumen treatment on rice husk highlighted the kinetic of lignocellulose degradation including the coefficient values. The assessment of Si release in liquid media was showing the potential of rumen-treated husk as high Si content solid fertilizer due to the highest release result. The result in Si extraction in alkaline solution suggested that the biological treatment can be applied as substitute for the thermal pretreatment in Si extraction with the common procedure. A study to observe the optimum concentration of organic acid in Si release, and the optimum of alkaline solution in Si extraction is highly recommended for the next investigation.

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Risk Assessment and Management Recommendations of Invasive Species in Papandayan Mountain Nature Reserve, West Java

Anggi Sella Nyuanti*, Rina Ratnasih Irwanto, Elham Sumarga

School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

*) Corresponding author; E-mail: anggisella22@gmail.com
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Abstract

Natural disasters and anthropogenic disturbances have caused several locations in the Papandayan Mountain Nature Reserve to be more vulnerable and exposed further triggering the presence of invasive species. This presence impacts the environment, the economy, and the health of humans and animals. This research aims to assess the risks of these invasive species, followed by various recommendation strategies towards their species management. Data on species diversity was collected by vegetation analysis, using the quadratic method by purposively sampling plots. Results showed the existence of six invasive species in Papandayan i.e. *Ageratina riparia* (Regel) R.M.King & H.Rob., *Ageratina adenophora* (Spreng.) R.M.King & H.Rob., *Austroeupatorium inulaefolium* (Kunth) R.M.King & H.Rob., *Imperata cylindrica* (L.) Raeusch., *Rubus moluccanus* L., and *Ageratum conyzoides* (L.) L.. The assessment of invasive species was assessed through two indices; Risk Index and Feasibility Index. Each of them was calculated based on the Risk Assessment Protocol to determine their strategy of control. Based on the Risk and Feasibility Indices, several recommendation strategies to manage invasive species are proposed.

Keywords: *Ageratina riparia*, invasive risk assessment, invasive species, Papandayan Mountain Nature Reserve

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1. Introduction

Papandayan Mountain Nature Reserve is a region that often experiences various natural disasters, such as volcanic eruptions and anthropogenic disturbances in the form of forest encroachment, opening up the environment to be more vulnerable [1]. These exposed conditions and scars of disturbance prove to be a suitable environment for the growth of invasive species [2]. Invasive species are native or non-native species that grow uncontrollably, have an effective way of seed dispersion and rapid reproduction, with their presence resulting on the disturbance of native species. [3]. In natural ecosystems, the presence of invasive species frequently results in negative impacts towards the environment, economy, and health of humans and animals [3, 4]. In Baluran National Park, East Java, *Acacia nilotica* has invaded almost 6,000 ha out of the 10,000 hectares of the savanna. The area has been converted into shrubs, causing a decrease in grass productivity for animal feed, and thus consequently threatening the lives of wildlife such as bull, buffalo, and deer [5]. In the United States, the impact of invasive species is expected to reach USD 167.9 billion [6]. Also, invasive species can cause health problems, for example, *Ambrosia artemisiifolia*, native in Central America, can cause sunburn on direct contact with humans [7].

The control of invasive species needs to be planned seriously to prevent the distribution of invasive species, especially to the natural ecosystems [8]. Several efforts in controlling invasive species have been implemented: mechanically, through felling or burning; chemical controls through the usage of natural herbicides; or, through biological controls, by bringing natural enemies. However, the results of these methods have been deemed ineffective, with staggeringly expensive costs [8]. The study of preventing the arrival of invasive foreign species is the most effective strategy compared to eradication [9]. Risk analysis is an effort to predict the chances of risk, distribution impacts, and threats to the preservation of natural resources due to the presence of invasive alien species [10]. Therefore, in this paper, conducting a review through risk assessments of invasiveness and management feasibility of invasive species is established as the basis for more effective and efficient management [10]. A risk assessment is needed to determine the risk of each invasive species, before being able to propose recommendations for appropriate invasive species management in Papandayan Nature Reserve.

2. Methods

2.1. Study Area and Sampling Time

The research was carried out in March until October 2019 in the Papandayan Mountain Nature Reserve, Garut, West Java. The research location is divided into three types of land cover, i.e. grassland (Pondok Salada and Tegal Alun), Tepi Kawah, and Cisupabeureum (Fig. 1).

2.2. Vegetation Analysis

The research was carried out in March until October 2019 in the Papandayan Mountain Nature Reserve, Garut, West Java. The research location is divided into three types of land cover, i.e. grassland (Pondok Salada and Tegal Alun), Tepi Kawah, and Cisupabeureum (Fig. 1).

Vegetation analysis was conducted to determine the invasive species, using the quadratic method. This was done by purposively sampling plots on three types of land cover, i.e. grassland, crater rim, and mixed forest. There were 18 plot of 5 x 5 m for the shrubs and 36 plots of 2 x 2 m for the herb. The composition of the invasive species in Papandayan was determined by the Index of Important Values (IV). The calculation of IV for herbs and shrubs were obtained by summing the relative density values and the frequency relative values, by using the following equation [11]:

$$\text{Density (D)} = \frac{\text{Number of individuals in the sample}}{\text{Total area of the sample}}$$

$$\text{Relative Density (RD)} = \frac{\text{Density of one species in the sample}}{\text{Total density of all species in the sample}} \text{ x } 100\%$$

$$\text{Frequency (F)} = \frac{\text{Number of sub-plots in which a species occurs}}{\text{Total number of sub-plots in sample}}$$

$$\text{Relative frequency (RF)} = \frac{\text{Frequency of a species}}{\text{Total frequency of all species in sample}} \text{ x } 100\%$$

The Important Value index (IV) of shrubs and herbs = RD + RF

2.3. The identification of Invasive Species

The specimens were identified based on Flora of Java book volume I-III [12-14]. Specimens not available in the book were identified based on local name references from Papandayan Mountain Nature Reserve officials and other

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references. Invasive species were classified by checking into online databases such as *Invasive Species Specialist Group* (ISSG), *Convention of Biological Diversity* (CBD), *Center for Agriculture Biosciences International* (CABI), *Guide Book to Invasive Species in Indonesia*, dan *Forest in Southeast Asia-Indonesia Program* (FORIS-INDONESIA).

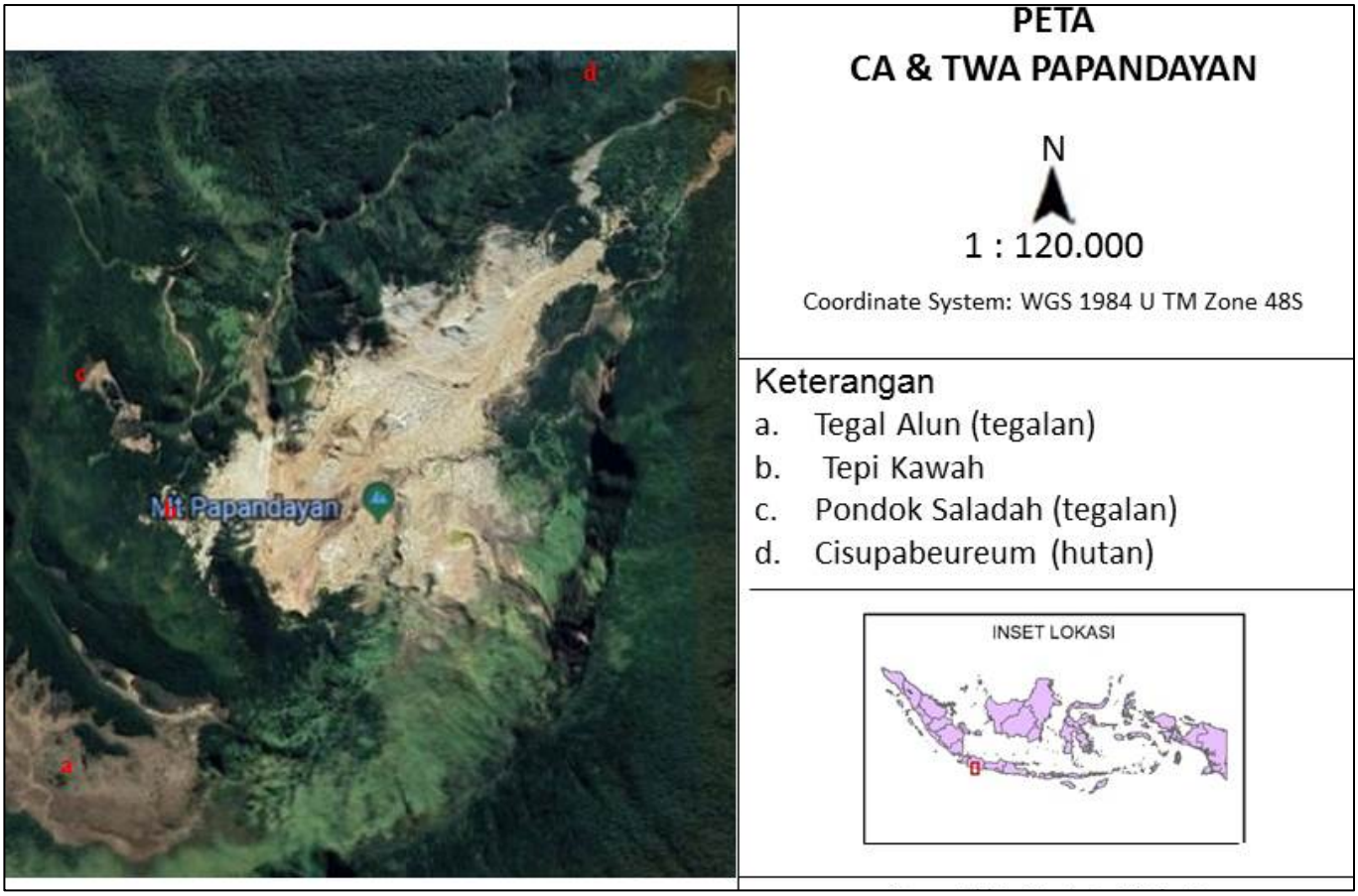

Figure 1 Research Site of Study (a) Tegal Alun (grassland), (b) Tepi Kawah (crater rim), (c) Pondok Saladah (grassland), (d) Cisupabeureum (mixed forest)

2.4. Risk Assessment

The research was carried out in March until October 2019 in the Papandayan Mountain Nature Reserve, Garut, West Java. The research location is divided into three types of land cover, i.e grassland (Pondok Salada and Tegal Alun), Tepi Kawah, and Cisupabeureum (Fig. 1).

A risk assessment of invasive species was analysed using The Invasive Species Risk Management System Protocol [15] modified by [10]. It was further adapted to suit the conditions of the Indonesian habitat. The risk assessment was further determined by three components, i.e. Risk Index, Feasibility Index, and Management Recommendation.

Risk index (R) was obtained after all questions from three related parameters were answered. The scores from each parameter were processed by the equation as follows [10]: **The risk index = I × Ip × DP [10].** Invasiveness (I), the total score is divided by 15 and multiplied by 10; Impact (Ip), the total score is divided by 19 and multiplied by 10; Distribution potential (DP), total score.

One of the parameters to obtain F (feasibility index) is the actual distribution (AD) of invasive species. The AD value was determined in percentage units using the following formula [16]:

$$\text{Percentage of AD} = \text{x } 100\% \frac{\sum_{j}^{i} FiAj}{\text{A PNR}} \text{ x}100\%$$

with, Fi = Frequency of species,
iAj = Area of ecosystem where species found (ha)
A PNR = Area of Papandayan Mountain Nature Reserve.

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F was obtained after answering all questions from three related parameters using formula as follows [10]: **The feasibility index = C × IPD × P**. Control Cost (C), the total score is divided by 15 multiplied by 10; Actual Distribution (AD), the total score is divided by 12 multiplied by 10; Persistence (P), the total score is divided 11 multiplied by 10. Risk Index and Feasibility index categorized as showed in Table 1 [10].

The R and F score obtained were classified into the following categories [10]: The higher the value of R showed a higher risk of invasive species, while the higher the value of F showed lower feasibility to manage invasive species. The recommended strategy of invasive species management was determined based on the matrix of score and category of R and F, as shown in Table 2 [10].

3. Results and discussion

3.1. Invasive Species in Papandayan Nature Reserve

There were six invasive species belong to three families of Asteraceae, Poaceae, and Rosaceae grow in Papandayan Nature Reserve (Table 3). The invasive species mostly grew in an open area of the grassland, but none in the vegetation at the edge of crater. The grassland area has been disturbed in the form of forest fires and has open vegetation cover so that 6 invasive alien species can be found in this area because invasive alien species are relatively easy to grow in the open and experience disturbance [3] The type of soil conditions in the edges of the dry craters was considered not suitable for invasive species to grow [10, 17]. One of the factors that prevent invasive alien species from growing and spreading is unsuitable abiotic conditions [17]. In Cisupabeureum forest area, only two invasive species, i.e. *A. riparia* and *A. adenophora* had grown because of the coverage of vegetation is difficult to make other species grow [3].

3.2. Invasive Species Risk Assessment

Based on the calculation of Risk Index, the invasive species in Papandayan was categorized as low risk (*A. conyzoides*) (4.18), to high risk (*A. riparia* (Regel) R.M.King & H.Rob.*)* (154.22) (Table 4). *A. riparia* has the highest Risk index, which means that its ability to invade a new habitat is considerably high. *A. riparia,* a shade-tolerant plant, can also appear on disturbed forest habitats [18, 19]. The species is also a prolific seeder and grows very fast compared to other invasive species [20-22]. Additionally, *A. riparia* can suppress the growth of other species due to its rapid and high-density growth, dominating ground covers among the locations [23]. In our research, *A. riparia* grew in a large area, and based on an estimated calculation, its potential distribution resulted in more than 50% of study area. The species was tolerant of shade. The branches of each individual *A. riparia* plants intertwined with each other, producing a blanket effect with 100% ground covers. This means that the seeds of other forest species may not be able to reach the soil to initiate germination [23]. Other invasive species that are not tolerant of shade require open land and high light intensity to grow such as *A. adenophora* can be established in open areas, forest areas, forest edges, and clearing disturbed areas [24].

The feasibility control of invasive species in Papandayan varied from the low to the high. The Feasibility Index of *A.riparia* was found to have the highest score (226.90), compared to other invasive species (Table 5). This indicates that the feasibility of management *A. riparia* in Papandayan is low, or substantially difficult to implement. This is could be related to *A. riparia* widespread distribution. In another study, the widespread growth species of *Dioscorea centruroides* had low management feasibility [16].

A. riparia was considered to spread widely in Papandayan. In our study, the species grew in three locations of sampling area, i.e Tegal Alun, Pondok Salada and Cisupabeureum. In contrast, other invasive species was usually only found in one or two sampling areas. Based on our research, the percentage distribution of *A. riparia* was predicted to be amounting to 50-60% of study area. Further *A. riparia* was found to produce an allelopathy compound i.e. phenolic *O-Coumaric acid (2-hydroxycoumaric),* which can inhibit the growth of other species [25, 26]. *A. adenophora* invaded 30-40% of land, mostly in open areas in grassland (Pondok Salada) and *A. inulaefolium* invaded 19% of the land. The number was only scattered in grassland areas *A. conyzoides* (1%), *I cylindrical* (9%) and *R. mollucanus* (24%) were found to be localized only in Pondok Salada. Control costs are not too high because the distribution is not too broad.

The traditional management of invasive species in Papandayan can be considered a solution. This is usually done through a mechanical control method, by slashing or cutting using a simple local equipment called *parang*. The predicted cost can amount to Rp 600,000,- per day. The costs were calculated based on the wages of labors at Rp. 100.000/day, multiplied by the number of workers, at six people.

Based on the matrix of recommendation management, Risk Index, and Feasibility Index, there are four recommendations for managing invasive species in Papandayan; managing its invasive species, managing the location of the growing place, performing limited action to the location of the growing invasive species; and lastly, monitoring (Table 6).

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Table 1 Classification of risk and feasibility indices [10]

Risk Score	Category	Feasibility Score	Category
>192	Very high	>113	Very low
101 – 192	High	56 – 112	Low
39 – 100	Hose	31 – 55	Medium
13 – 38	Low	14 – 30	High
< 13	Very low	<14	Very High

Table 2 Matrix of Invasive Species Recommendations of Management [10]

Risk Index	Feasibility Index				
	Very low >113	Low 56-112	Medium 31-55	High 14-30	Very high <14
Very low <14	Limited action	Limited action	Limited action	Limited action	Monitoring
Low 15-38	Limited action	Limited action	Limited action	Monitoring	Monitoring
Medium 39-101	Manage location	Manage location	Manage location	Protect location	Contain spread
High 102-192	Manage species	Manage species	Protect location	Contain spread	Destroy infestation
Very high >192	Manage species	Protec location and manage species	Contain spread	Destroy infestation	Eradicate

Table 3 Index Value (IV) of Invasive Species in Papandayan Nature Reserve

Species	Family	IV (%)		
		Grassland	Crater side	Forest
Ageratina riparia (Regel) R.M.King & H.Rob.	Asteraceae	56.50	-	58.40
Ageratina adenophora (Spreng.) R.M.King & H.Rob.	Asteraceae	51.71	-	43.20
Rubus moluccanus L.	Rosaceae	22.92	-	-
Austroeupatorium inulaefolium (Kunth) R.M.King & H.Rob.	Asteraceae	19.70	-	-
Imperata cylindrica (L.) Raeusch	Poaceae	16.70	-	-
Ageratum conyzoides (L.) L.	Asteraceae	1.33	-	-

Table 4 Risk Index and Category of Invasive Species in Papandayan Nature Reserve

Species	Parameters			Risk Index	Risk Category
	Invasiveness	Impact	Potential Distribution		
A. riparia	7.33	2.63	8	154.22	High
A. adenophora	6	2.63	4	63.12	Medium
I. cylindrical	5.33	2.63	1	14.01	Very low
R. mollucanus	5.33	1.05	2	11.19	Very low
A. inulaefolium	5.33	1.05	2	11.19	Very low
A. conyzoides	5.33	1.57	0.5	4.18	Very low

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Table 5 Feasibility Index and Category of Invasive Species in Papandayan Nature Reserve

Species	Parameters			Feasibility Index	Feasibility Category
	Control Cost	Distribution	Persistence		
A. riparia	6	6.66	4.54	182.21	Very low
A. adenophora	4.67	5	5.45	127.25	Very low
R. mollucanus	3.33	3.33	4.54	50.34	Medium
I. cylindrica	4	1.67	4.54	30.32	High
A. inulaefolium	4	0.83	4.54	15.07	High
A. conyzoides	3.33	0.41	3.63	4.95	Very high

Table 6 Recommendation of Invasive Species Management in Papandayan Nature Reserve

Species name	Risk Category	Feasibility Category	Recommendation of Management
A. conyzoides	Very low	Very high	Monitoring
I. cylindrical	Very low	High	Limited action
R. mollucanus	Very low	High	Limited action
A. inulaefolium	Very low	High	Limited action
A. riparia	High	Very low	Manage species
A. adenophora	Medium	Low	Manage location

Management recommendation to manage invasive species for *A. riparia* is aimed to reduce the environmental, economic, and social impacts of invasive species. This particular management approach is recommended by [10] especially for invasive species with high risk categories. The control feasibility of *A. riparia* is considered significantly low, due to its already-expanding distribution, thus causing considerable difficulties to control. Mapping of priority management locations can serve as a solution. This is done by modelling the distribution of species to configure the main environmental factors which affects the invasive species distribution [27, 28]. Another way is through mechanical controls, such as physically uprooting small plants and disposing them either by before the flowering of *A. riparia*. Cultivation, grubbing, hoeing, burning, replanting competitive pastures, or replacing *A. riparia* with native species can also prevent re-infestations [29]. Another management effort is to regularly monitor the cleaned areas every month [10].

Management location is recommended for *A. adenophora* aim to maintain environmental conditions of the conservation area [10]. This recommendation focuses more on location management, due to the lower potential distribution of *A. adenophora*. Mapping out the distribution of *A. adenophora* in Papandayan can be implemented [10, 15]. Management can also be done by slashing the seeds and flowers as they emerge, as well as clearing of colonization areas [22]. Utilization of biological control agents can be implemented by using the *Procecidocares utilis* flies and the *Eupatorii cercospora* mushrooms, considered as natural enemies to inhibit the growth of *A. adenophora* [30,31]. Limiting the invaded locations of *A. adenopora* from human access is also needed. This reduces the potential of *A. adenophora* being carried by human clothing and footwear to other places [23].

The recommended management of *I. cylindrica, A. inulaefolium,* and *R. mollucanus* is to perform limited actions aimed at reducing potential distribution invasive species to all research locations. This can be done by collecting the species bio-ecological data and analyze its response to management, to determine the best time for control [27]. Another method is by periodically monitoring the distribution of three species in Papandayan [10,15].

The monitoring recommendation for *A. conyzoides* is to detect changes in the risk of invasive species [10]. Generally, monitoring applies to species that have high management feasibility but with a low risk, therefore it does not necessarily need to be eradicated. Monitoring efforts can be implemented by making a permanent sample plot to monitor the spread of *A. conyzoides* [25], or by reviewing the invasiveness changes of *A. conyzoides* through a risk assessment within a certain period [32].

The recommendations above require continual monitoring efforts to detect threats of invasive species at the earliest possible time and evaluate any action that has been taken. These recommendations can become the foundational effort to control invasive species in Papandayan. This will further protect conservation areas in West Java. The role, support, and awareness of the threat of invasive species from various parties are needed. Therefore, dissemination, information exchange, and transfer of knowledge about invasive species

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are significantly important efforts in preventing the spread of invasive species in Papandayan Nature Reserve.

4. Conclusion

There are six invasive species within the area of the Papandayan moor i.e *Ageratina riparia Ageratina adenophora, Austroeupatorium inulaefolium, Imperata cylindrica, Rubus mollucanus* and *Ageratum conyzoides.* Recommendations towards the management of invasive species in Papandayan Nature Reserve depends on the character of each species. *A. riparia* is managed by managing the invasiveness of the species. *A. adenophora* is managed through the location its growing location. The management of *I. cylindrica, R. mollucanus* and*, A inulaefolium* is focused on limited actions. Finally, *A. conyzoides* can be managed effectively by periodical monitoring. *A. riparia* will need to be managed in the highest priority in Papandayan Nature Reserve, due to the highest risk of invasiveness (154.22), with low feasibility of control (182.21).

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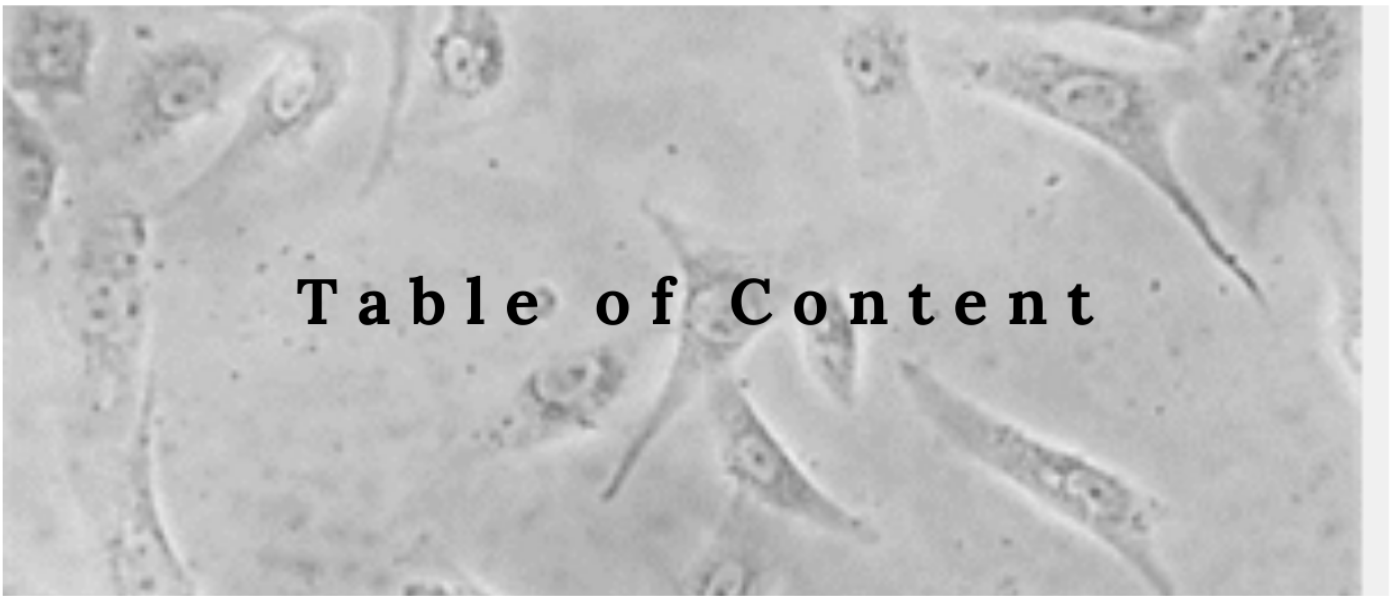

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