Table of contents

Volume 236

2019

Previous issue Next issue

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Preface

Papers

Ammonia-eliminating potential of *Gracilaria* sp. And zeolite: a preliminary study of the efficient ammonia eliminator in aquatic environment

M R Royan, M H Solim and M B Santanumurti

+ Open abstract View article PDF

OPEN ACCESS 012003

Identification of extracellular enzyme-producing bacteria (proteolytic, cellulolytic, and amylolytic) in the sediment of extensive ponds in Tanggulrejo, Gresik

OA Artha, Sudarno, H Pramono and LA Sari

+ Open abstract View article PDF

OPEN ACCESS 012004

Addition of water from the treatment pond of pangasius fillet waste (*Pangasius* sp.) with different concentrations in the cultivation medium due to the population growth of *Daphnia* sp.

H P Alvian, E D Masithah and M H Azhar

+ Open abstract View article PDF

OPEN ACCESS 012005

The growth and survival rate in lettuce aquaponic systems (*Latuca sativa*) of eels in various stocking densities of eel (*Monopterus albus*)

N K Portalia, L Sulmartiwi and B S Rahardja

+ Open abstract View article PDF

OPEN ACCESS 012006

The prevalence of benedeniasis in humpback grouper (*Cromileptes altivelis*) in floating net cages in Situbondo Regency, East Java, Indonesia

S O Wijaya, S Subekti and Kismiyati

+ Open abstract View article PDF

OPEN ACCESS 012007

The prevalence of fungi on groupers (*Epinephelus* sp.) in cage mariculture systems of the northern coast of Surabaya, East Java

E Yuliastuti, R Kusdawarti and Sudarno

+ Open abstract View article PDF

OPEN ACCESS 012008

The spectrum of light and nutrients required to increase the production of phycocyanin *Spirulina platensis*

H A Wicaksono, W H Satyantini and E D Masithah

+ Open abstract View article PDF

OPEN ACCESS 012009

The growth and survival rate of the larvae of the sunu grouper (*Plectropomus leopardus*) in different temperatures

L Lutfiyah, D S Budi and M F Ulkhaq

+ Open abstract View article PDF

OPEN ACCESS 012010

Preservation of common carp (*Cyprinus carpio*) sperm using 0.9% NaCl and ringer's lactate solution

D S Budi, L A Adawiyah and L Lutfiyah

+ Open abstract View article PDF

OPEN ACCESS 012011

Comparison of the efficiency (flash point, freezing point, and viscosity test) of biodiesels from *Sargassum* sp.

M B Santanumurti, M R Royan, S H Samara, S Sigit and M A Alamsjah

+ Open abstract View article PDF

OPEN ACCESS 012012

Study of patterns in the relationship of ecdysis with the age of freshwater crayfish *Cherax quadricarinatus* aged 76 Days

A H Fasya

+ Open abstract View article PDF

OPEN ACCESS 012013

Maximum density in the *Moina macrocopa* culture able to produce parthenogenesis in female offspring

A S Mubarak, D Jusadi, M Z Junior and M A Suprayudi

+ Open abstract View article PDF

OPEN ACCESS 012014

The oxygen content and dissolved oxygen consumption level of white shrimp *Litopenaeus vannamei* in the nanobubble cultivation system

D P Galang, A K Ashari, L Sulmatiwi, G Mahasri, Prayogo and LA Sari

+ Open abstract View article PDF

OPEN ACCESS 012015

The effect of the epiphytes of *Chaetomorpha crassa* on the total chlorophyll-a and growth of *Gracilaria verrucosa*

A L L Handayani, R J Triastuti and L Sulmartiwi

+ Open abstract View article PDF

OPEN ACCESS 012016

Growth monitoring of koi fish (*Cypri nus carpio*) in natural hatchery techniques in Umbulan, Pasuruan, East Java

F P Putri and N N Dewi

+ Open abstract View article PDF

OPEN ACCESS 012017

Dynamic Ratio Correlation of N:P in relation to the Diatom Abundance in the Intensive System of the Vannamei (*Litopenaeus vannamei*) Shrimp Pond

E D Masithah, D D Nindarwi, T Rahma and dan R R Satrya P I

+ Open abstract View article PDF

OPEN ACCESS 012018

Dynamic ratio correlation of N:P on the abundance of Bluegreen algae in an intensive system in a white shrimp (*Litopenaeous vannamei*) pond

E D Masithah, D D Nindarwi, A L A Suyoso and D Husin

+ Open abstract View article PDF

OPEN ACCESS 012019

Dynamic ratio correlation of N:P toward phytoplankton explosions in intensive systems of white shrimp pond

E D Masithah, D D Nindarwi, D Husin and T Rahma

+ Open abstract View article PDF

OPEN ACCESS 012020

Development of water and nutrient management models to improve multitrophic seafarming productivity

J A Surbakti, I A L Dewi, M A Alamsjah and M Lamid

+ Open abstract View article PDF

OPEN ACCESS 012021

Pond soil characteristic in reclaimed tidal lowlands and its correlation with the water quality for aquaculture

M Fitrani, I Wudtisin, M Kaewnern and R H Susanto

+ Open abstract View article PDF

OPEN ACCESS 012022

The dynamics of total organic matter (tom) on sangkuriang catfish (*clarias gariepinus*) farming at upt ptpbp2kp and the effectiveness of freshwater bivalve (*anodonta woodiana*) in reducing the total organic matter with varying density

D Arfiati, C D G Putra, A H Tullah, S W A Permanasari and A W Puspitasari

+ Open abstract View article PDF

OPEN ACCESS 012023

Growth and morphological changes in relation to the maturation of male Japanese eel, *Anguilla japonica* injected with human chorionic gonadotrophin (HCG) in the different interval in the tropical region

Y T Hee, F F Ching and S Senoo

+ Open abstract View article PDF

OPEN ACCESS 012024

Genetic diversity of the endangered species *Sphyrna lewini* (Griffith and Smith 1834) in Lombok based on mitochondrial DNA

S Hadi, N P Anggraini, E Muttaqin, B M Simeon, B Subhan and H Madduppa

+ Open abstract View article PDF

OPEN ACCESS 012025

Sex ratio and size at first maturity of razor clam *Solen* sp. in Pamekasan and Surabaya coastal area, East Java, Indonesia

N Trisyani, N I Wijaya and I Yuniar

+ Open abstract View article PDF

OPEN ACCESS 012026

Improving productivity and water quality of catfish, *Clarias* sp. cultured in an aquaponic ebb-tide system using different filtration

E Setiadi, I Taufik, Y R Widyastuti, I Ardi and D Puspaningsih

+ Open abstract View article PDF

OPEN ACCESS 012027

Different substrate of trickling filter on growth, survival rate, and water quality of common carp (*Cyprinus carpio*) cultivation by using an intensive recirculation system

E Setiadi, I Taufik, Y R Widyastuti, I Ardi and A Saputra

+ Open abstract View article PDF

OPEN ACCESS 012028

Water quality dynamic, production and profitability of catfish, *Clarias* sp. cultured at different design construction of aquaponic

Y R Widyastuti, E Setiadi, I Taufik and L Setijaningsih

+ Open abstract View article PDF

OPEN ACCESS 012029

Effect of C:N ratio on the spore production of *Bacillus* sp. indigenous shrimp pond

A Yuniarti, N B Arifin, M Fakhri and A M Hariati

+ Open abstract View article PDF

OPEN ACCESS 012030

Comparative Test on Bacteria in the Digestive Tract of Vannamei Shrimp (*Litopenaeus vannamei*) at Intensive and Extensive Ponds in Ujungpangkah, Gresik

D Ningrum, M Arief and dan K T Pursetyo

+ Open abstract View article PDF

OPEN ACCESS 012031

Growth Performance of Laboratory-Scale *Chaetoceros calcitrans* in Different Containers

M Jannah, M F Ulkhaq, M H Azhar, Suciyono and dan W Soemarjati

+ Open abstract View article PDF

OPEN ACCESS 012032

Study of the Dynamic Density and Diversity of Plankton at Different Brackishwater Pond Managements in Gresik, East Java

O Tilahwatih, E D Masithah and dan B S Rahardja

+ Open abstract View article PDF

OPEN ACCESS 012033

The Effect of Demineralization Stage of Agar's Solid Waste on the Characterization of Activated Carbon

R Febrianto, Sudarno and R Kusdarwati

+ Open abstract View article PDF

OPEN ACCESS 012034

The Dynamic of Density and Diversity of Cyanophyta in Different Pond Bases in Educational Pond of Faculty of Fisheries and Marine Universitas Airlangga

S Z Cahyani, E D Masithah and Prayogo

+ Open abstract View article PDF

OPEN ACCESS 012035

Effect of Different Salinity Level within Water Against Growth Rate, Survival Rate (FCR) of Catfish (*Clarias* sp.)

D Prananingtyas, Prayogo and S Rahardja

+ Open abstract View article PDF

OPEN ACCESS 012036

Molecular identification and phylogenetic reconstruction of two fiddler crabs (*Uca forcipata* and *Uca triangularis*)

S Andriyono, H Pramono and H W Kim

+ Open abstract View article PDF

OPEN ACCESS 012037

The molecular identification and phylogenetic reconstruction of Palaemonid and Penaeid shrimp from the southern part of Bangladesh

M J Alam, S Andriyono, A T M Eunus and H W Kim

+ Open abstract View article PDF

OPEN ACCESS 012038

Morphometric characteristics of Fur Cockles (*Anadara spp.*) in Wonokromo and Juanda Estuary, Surabaya

P B Pamungkas, K T Pursetyo, J Triastuti and N N Dewi

+ Open abstract View article PDF

OPEN ACCESS 012039

Stock status of ark clams (*Anadara spp.*) based on dredge fishing of the east coast of Surabaya, Indonesia

N N Dewi, K T Pursetyo, O P Darmono, F R Fachri, F S Puspitasari and A Damora

+ Open abstract View article PDF

OPEN ACCESS 012040

The distribution patterns and biomass of bivalves in Segoro Tambak estuary, Sedati, Sidoarjo, East Java

S H Liyana, L A Sari, N N Dewi, E D Masithah, A M Sahidu and K T Pursetyo

+ Open abstract View article PDF

OPEN ACCESS 012041

Inventorization of reef fish on Tabuhan Island, Banyuwangi, East Java, Indonesia

Suciyono, M A Azhar, M F Ulkhaq and H Kenconojati

+ Open abstract View article PDF

OPEN ACCESS 012042

Dynamic study on the effect of calcium hydroxide and sodium bicarbonate treatment on the N/P ratio and plankton abundance

M R N Tsany, E D Masithah, B S Rahardjo and D D Nindarwi

+ Open abstract View article PDF

OPEN ACCESS 012043

Distribution patterns and the biomass of bivalves at Segoro Tambak estuary, Sedati, Sidoarjo, East Java

S H Liyana, L A Sari, N N Dewi, E D Masithah, A M Sahidu and K T Pursetyo

+ Open abstract View article PDF

OPEN ACCESS 012044

Optimization of diatom *Haslea ostrearia* cultivation in different mediums and nutrients

S Arsad, C Stavrakakis, V Turpin, P Rossa, Y Risjani, L A Sari, F S Prasetiya and J-L Mouget

+ Open abstract View article PDF

OPEN ACCESS 012045

Coastal ecosystem model based on environmental suitability and carrying capacity of the fishpond in Banyuwangi Region, East Java, Indonesia

E W Setyaningrum, Maghdalena, A T K Dewi, M. Yuniartik and E D Masithah

+ Open abstract View article PDF

OPEN ACCESS 012046

Development and succession of sessile macrofouling organisms on the artificial structure in the Shallow Coastal Waters of Sabah, Malaysia

M A M Affandy, J Madin, K P Jakobsen and M Auluck

+ Open abstract View article PDF

OPEN ACCESS 012047

Fish species difference around the light of metal halide lamps and LED lamps with mini purse seine operation

M A Sofijanto, D Arfiati, T D Lelono and A Muntaha

+ Open abstract View article PDF

OPEN ACCESS 012048

Status of coral diseases and compromised health syndromes on Pemuteran shallow reefs, North Bali island

W Karim

+ Open abstract View article PDF

OPEN ACCESS 012049

Management options for restoring artificial coral reefs in Indonesia: strengthening in institutional approach

Rudianto and Ahmad Zainul

+ Open abstract View article PDF

OPEN ACCESS 012050

Growth of salt-secretor and non-salt secretor mangrove seedlings with varying salinity and their relations to habitat zonation

M Basyuni, Ramayani, A Hayullah, Prayunita, M Hamka, L A Putri and S Baba

+ Open abstract View article PDF

OPEN ACCESS 012051

Distribution of *Ctenactic Echinata* and *Fungia Consinna* coral on Mamburit island, Sumenep regency, East Java

Sawiya, D Arfiati, Guntur and U Zakiyah

+ Open abstract View article PDF

OPEN ACCESS 012052

Diversity species and condition of seagrass ecosystem in Teluk Awur and Prawean Jepara

I Riniatsih, A Ambariyanto, E Yudiati, R Hartati, W Widianingsih and R T Mahendrajaya

+ Open abstract View article PDF

OPEN ACCESS 012053

Copper (Cu) and Cadmium (Cd) toxicity on growth, chlorophyll-a and carotenoid content of phytoplankton *Nitzschia* sp

D Hindarti and A.W. Larasati

+ Open abstract View article PDF

OPEN ACCESS 012054

Vegetation Characteristic and Micro Environment of Mangrove Rehabilitation Forest at Coastal Areas of East Sinjai, South Sulawesi

H Setiawan

+ Open abstract View article PDF

OPEN ACCESS 012055

The Use of Water Lettuce (*Pistia stratiotesas*) as Phytoremediator for Concentration and Deposits of Heavy Metal Lead (Pb) Tilapia (*Oreochromis niloticus*) Gills

A A D Amalia, B S Rahardja and Rr J Triastuti

+ Open abstract View article PDF

OPEN ACCESS 012056

The Effectiveness of Heavy Metals Pb, Cd and Zn Reduction in NPK Fertilizer Waste Combined with Biofilters of Seaweed (*Gracillaria* sp.), Blood Clam (*Anadara* sp.), and Zeolite

A R K Sari, R K Harryes, F A Anggraini, M A Alamsyah and dan A Ahadi

+ Open abstract View article PDF

OPEN ACCESS 012057

The Effectiveness of Combining *Gracilaria* Sp. Seaweed Biofilter and *Anadara granosa* Shell with Zeolite in the Decrease in the Level of Mercury (Hg) Heavy Metal

J A Spespatri, B S Rahardja and A A Abdillah

+ Open abstract View article PDF

OPEN ACCESS 012058

The Effectiveness of Combination of Seaweed (*Gracillaria* sp.), Blood Clamp (*Anadara granosa*), and Zeolite as Biofilter in the Reduction of Heavy Metal Copper (Cu)

I Aduma, B S Rahardja and A A Abdillah

+ Open abstract View article PDF

OPEN ACCESS 012059

Bioaccumulation of Cadmium (Cd) Heavy Metal on Seaweed (*Gracilaria* sp.) in Traditional Fishpond of Jabon Subdistrict, Sidoarjo District

O Ardiyansyah, Sudarno and Rosmanida

+ Open abstract View article PDF

OPEN ACCESS 012060

Identification of Proteolytic Bacterial Isolates in Sediment Ecosystem of Gunung Anyar Mangrove Forest, Surabaya

P B Utomo, Sudarno and B S Rahardja

+ Open abstract View article PDF

OPEN ACCESS 012061

The Analysis of Cockle (Anadara inaequivalvis) Gonad Maturity Level in the Estuary of Banjar Kemuning River, Sedati, Sidoarjo

R F Saputra, E D Masithah and P D Wulansari

+ Open abstract View article PDF

OPEN ACCESS 012062

Spatial and Temporal Variation of Biomass Blood Cockle (*Anadara* sp.) in Estuaries Dadapan, Sedati Sub-District, Sidoarjo, East Java

M R Ramadhan, K T Pursetyo, Prayogo and N N Dewi

+ Open abstract View article PDF

OPEN ACCESS 012063

Distribution Patterns and Biomass of Bivalve in Juanda and Segoro Tambak Estuary in Sedati, Sidoarjo, East Java

S H Liyana, E D Masithah and A M Sahidu

+ Open abstract View article PDF

OPEN ACCESS 012064

Analysis of Cadmium (Cd) Heavy Metal on Sediment and Mangrove Leaves *Avicennia marina* at Mangrove Ecotourism Wonorejo, Surabaya

W C Dermawan, Prayogo and B S Rahardja

+ Open abstract View article PDF

OPEN ACCESS 012065

Analysis of Lead (Pb) Value Comparison on Seaweed (*Eucheuma cottonii*) in Bluto and Saronggi Sumenep Marine, Madura, East Java

N L Komariyah, K T Pursetyo and S A Sudjarwo

+ Open abstract View article PDF

OPEN ACCESS 012066

The potential addition of lemuru oil to commercial feed to increase the content of EPA and DHA in eels (*Monopterus albus*)

A Imanisa, M B Santanumurti, M Lamid and Agustono

+ Open abstract View article PDF

OPEN ACCESS 012067

Effect of lysine in addition to commercial feed on crude protein and the energy digestibility of gourami (*Osphronemus gouramy*)

D Setiyawan, S H Samara, Agustono and M A A Arif

+ Open abstract View article PDF

OPEN ACCESS 012068

Addition of the papain enzyme to commercial feed against protein retention and feed efficiency in eels (*Anguilla bicolor*)

DA Liono, M Arief, Prayogo and W Isroni

+ Open abstract View article PDF

OPEN ACCESS 012069

Combination of papain enzyme and phytase enzyme in commercial feed and the protein and energy retention of tilapia *Oreochromis niloticus*

A Saifulloh, M B Santanumurti, M Lamid and W P Lokapirnasari

+ Open abstract View article PDF

OPEN ACCESS 012070

The effect of giving cake artificial feed on the survival rate, and growth of Common carp (*Cyprinus carpio*) larva in an Installation of Freshwater Culture (IBAT) in Punten, Batu.

I P Zainiyah, Rozi, W H Satyantini and A M Sahidu

+ Open abstract View article PDF

OPEN ACCESS 012071

The Utilization of Phytase Enzymes and SEM Analysis in order to increase the Quality of Rice Bran as a Layer and Fish Feed

M Lamid, Anam Al-Arif and S H Warsito

+ Open abstract View article PDF

OPEN ACCESS 012072

The dynamic relationship of phytoplankton abundance and diversity in relation to white shrimp (*Litopenaeus vannamei*) feed consumption in intensive ponds

D D Nindarwi, E D Masithah, D Zulian and A LA Suyoso

+ Open abstract View article PDF

OPEN ACCESS 012073

Effect of partial replacement of fish meal with *Spirulina platensis* meal in practical diets and culture location on growth, survival, and color enhancement of percula clownfish *Amphiprion percula*

S Hudaidah, B Putri, S H Samara and Y T Adiputra

+ Open abstract View article PDF

OPEN ACCESS 012074

The Effect of Different level of Probiotic Addition on Commercial Feed against Digestibility and Efficiency of Nile Tilapia Feed (*Oreochromis Niloticus*)

D Taufik, M Arief and H Kenconojati

+ Open abstract View article PDF

OPEN ACCESS 012075

The Effect of Adding Synbiotics Into Commercial Feed Towards Protein Retention and Fat Retention of Dumbo Catfish (*Clarias* sp.)

H Syevidiana, M Arief and I S Hamid

+ Open abstract View article PDF

OPEN ACCESS 012076

The Effect of Adding Lysine in Commercial Feed on Growth Rate, Feed Efficiency, and Feed Convertion Ratio to Tambaqui (*Colossoma Macropomum*)

L V D Putra, U Agustono and S H Kenconojati

+ Open abstract View article PDF

OPEN ACCESS 012077

The Effect of Coconut Shell Liquid Smoke in Commercial Feed on Total Bacteria of *Pseudomonas Aeruginosa* in the Tilapia's Kidney (*Orechromis niloticus*)

M Rahmawati, Sudarno and S Subekti

+ Open abstract View article PDF

OPEN ACCESS 012078

The Effect of Coconut Shell Liquid Smoke in Commercial Feed Towards Total *Pseudomonasaeruginosa* Bacteria on Gastrointestinal Tract Tilapia (*Oreochromis Niloticus*)

S Rahmadini, Sudarno and S Subekti

+ Open abstract View article PDF

OPEN ACCESS 012079

Antibacterial activity of honey in preserving high-pressure cooked milkfish stored at room temperature

D A Hakim, W Tjahjaningsih and Sudarno

+ Open abstract View article PDF

OPEN ACCESS 012080

Bacterial composition in the gastrointestinal tract of *Uca* spp crabs fed on *Avicennia marina* leaf litter

M A B Kareho, E D Masithah and W Tjahjaningsih

+ Open abstract View article PDF

OPEN ACCESS 012081

The correlation between ectoparasite infestation and the total plate count of *Vibrio* sp. in pacific white shrimp (*Litopenaeus vannamei*) in ponds

G Mahasri, Rozi, A T Mukti, W H Satyantini and N M Usuman

+ Open abstract View article PDF

OPEN ACCESS 012082

In vitro study of an ethanolic extract of coffea leaves to inhibit freshwater pathogenic bacteria

H Kenconojati, MF Ulkhaq, DS Budi and MH Azhar

+ Open abstract View article PDF

OPEN ACCESS 012083

The effect of noni fruits (*Morinda citrifolia*) with different ripeness stages against the total erythrocytes and leukocytes of comet goldfish (*Carassius auratus*) infested by *Argulus*

E Setyaningsih, Kismiyati and S Subekti

+ Open abstract View article PDF

OPEN ACCESS 012084

The protection capacity of the crude and whole protein spores of *Myxobolus koi* as an immunostimulant material development in goldfish (*Cyprinus carpio*) for preventing Myxobolusis

G Mahasri, M Yusuf, R Woro and M B Santanumurti

+ Open abstract View article PDF

OPEN ACCESS 012085

Identification of white spot syndrome virus (WSSV) in pacific white shrimps (*Litopenaeus vannamei*) from ponds postexposure to immunogenic membrane proteins (*Zoothamnium penaei*)

P A Wiradana, G Mahasri, R E R Sari, U C Marwiyah and R Prihadhana

+ Open abstract View article PDF

OPEN ACCESS 012086

Gills and swimming leg histopathologies in pacific white shrimp (*Lithopenaeus vannamei*) from ponds exposed to the immunogenic membrane proteins of *Zoothamnium penaei*

R E R Sari, G Mahasri, P A Wiradana and U C Marwiyah

+ Open abstract View article PDF

OPEN ACCESS 012087

Total plate count and identification of vibrio in pacific white shrimp (*Litophenaeus vannamei*) from ponds and in those exposed to immunogenic protein membrane *Zoothamnium penaei*

U C Marwiyah, G Mahasri, R E Ratnasari and P A Wiradana

+ Open abstract View article PDF

OPEN ACCESS 012088

Effectiveness of *Nitrobacter* on the specific growth rate, survival rate and feed conversion ratio of dumbo catfish *Clarias* sp. with density differences in the aquaponic system

A S Taragusti, M B Santanumurti, B S Rahardja and Prayogo

+ Open abstract View article PDF

OPEN ACCESS 012089

The erythrocyte and leucocyte profile of saline tilapia (*Oreochromis Niloticus*) in a cultivation system with nanobubbles

M Gunanti, P D Wulansari and K Kinzella

+ Open abstract View article PDF

OPEN ACCESS 012090

The prevalence and intensity of ectoparasites infecting vanname shrimp (*Litopenaeus vannamei*) reared in different ponds

N Nurlatiffah, Kismiyati and MF Ulkhaq

+ Open abstract View article PDF

OPEN ACCESS 012091

Protozoan parasites of Vannamei Shrimp (*Litopenaeus vannamei*) in farmed fish from Pasuruan, Indonesia

U Hafidloh and P D W Sari

+ Open abstract View article PDF

OPEN ACCESS 012092

Growth of *Bacillus* sp. and *Flavobacterium* sp. in culture media with the addition of liquid whey tofu waste

W H Satyantini, R M Pratiwi, A M Sahidu and D D Nindarwi

+ Open abstract View article PDF

OPEN ACCESS 012093

The effect of noni *Morinda citrifolia* L. fruit extracts on the gill histopathological changes of Nile tilapia *Oreochromis niloticus*

A T Mukti, E Dewi, W H Satyantini, L Sulmartiwi, Sudarno and M Hassan

+ Open abstract View article PDF

OPEN ACCESS 012094

Prevalence and intensity of ectoparasites in Pacific white shrimp (*Litopenaeus vannamei*) seeds from a pond and hatchery

G Mahasri, T Hidayat and Sudarno

+ Open abstract View article PDF

OPEN ACCESS 012095

Prevalence and intensity of ectoparasites in gabus fish (*Channa striata*) at Cangkringan Fishery Cultivation Technology Development Center, Sleman, Yogyakarta

E N Fitriani, Rozi, M Arief and H Suprapto

+ Open abstract View article PDF

OPEN ACCESS 012096

Antibacterial activity of bitter gourd (*Momordica charantia l.*) leaf extract against *Aeromonas hydrophila*

D A Masithoh, R Kusdarwati and D Handijatno

+ Open abstract View article PDF

OPEN ACCESS 012097

Determination of the *aerolysin* gene in *Aeromonas hydrophila* using the polymerase chain reaction (pcr) technique

G Christy, R Kusdawarti and D Handijatno

+ Open abstract View article PDF

OPEN ACCESS 012098

Bioaccumulation and histopathological effect on the gills and liver of silver barb (*Barbonymus gonionotus*) exposed to the heavy metal nickel

I Purwanti, W Arroisi, B S Rahardja and L Sulmartiwi

+ Open abstract View article PDF

OPEN ACCESS 012099

Identification and prevalence of the ectoparasite *Octolasmis* in sand lobster (*Panulirus homarus*) and bamboo lobster (*Panulirus versicolor*) in Floating Net Cages in Sape, Bima Regency, West Nusa Tenggara Province, Indonesia

L. Yusgita, Kismiyati, S. Subekti, P D Wulansari and M K Amiin

+ Open abstract View article PDF

OPEN ACCESS 012100

Treatment of *Chlorella sp*. extract on heat shock cluster (HSC) response from the tissue and bloodcells proliferation of *Epinephelus fuscoguttatus-lanceolatus* infected by *Viral Nervous Necrosis*

U Yanuhar, I Al-Hamidy and N R Caesar

+ Open abstract View article PDF

OPEN ACCESS 012101

Superdioxide dismutase (SOD) and metallothionein (MT) *Tubifex tubifex* at the acute mercury exposure

I M Widiastuti, A M S Hertika, M Musa and D Arfiati

+ Open abstract View article PDF

OPEN ACCESS 012102

Antifungal activity of marine sponges (Class Demospongiae) collected from Biak, Indonesia

R D Kasitowati, K Wittriansyah, A Trianto, D C Pratiwi and M A P Panjaitan

+ Open abstract View article PDF

OPEN ACCESS 012103

Antibacterial activities of *Physalis angulata* herb extract on white feces diseases (WFD) in *Litopenaeus* shrimp *vannamei*

E Saraswati and AS Wijaya

+ Open abstract View article PDF

OPEN ACCESS 012104

The Effects of Mercury Chloride (Hgcl2) on the Changes in Hematology and Blood Sugar Level in Carps (*Cyprinus carpio*)

I Setiyowati, H Suprapto and G Mahasri

+ Open abstract View article PDF

OPEN ACCESS 012105

Change in Two-Spot Catfish Histopathological Liver (Mystus nigriceps) Accumulated with Heavy Metal Cadmium (Cd) in Ketingan Estuary, Sidoarjo - East Java, Indonesia

L Anggitasari, H Suprapto and dan D D Nindarwi

+ Open abstract View article PDF

OPEN ACCESS 012106

Effectiveness of Pepaya Leaf Extract (*Carica Papaya* L.) to Control Ectoparasite *Argulus* on Common Carp (*Cyprinus Carpio*)

L S Azizah, Kismiyati and A H Fasya

+ Open abstract View article PDF

OPEN ACCESS 012107

Bacterial Identification from Marine Ornamental Fish in Fish Quarantine, Quality Control and Fishery Products Safety Class I Denpasar, Bali

N R Rukmana, G Mahasri, S N Hidayah, M F Ulkhaq and H Kenconojati

+ Open abstract View article PDF

OPEN ACCESS 012108

Prevalence and Intensity of Ectoparasites of Tilapia (*Oreochromis niloticus*) in Ponds with Low, Medium and High Stocking Density

M Indahsari, Kismiyati and M F Ulkhaq

+ Open abstract View article PDF

OPEN ACCESS 012109

Texture profile of the bread produced from composite flour *Bruguiera gymnorrhiza* flour (BGF) and wheat flour

M N G Amin, M N Hasan, Zakariya, S A Pralebda, H Pramono, E Saputra, S Subekti and M A Alamsjah

+ Open abstract View article PDF

OPEN ACCESS 012110

The potential of seaweed waste (*gracilaria* sp. and *eucheuma cottonii*) as a medium density fiberboard (mdf)-based pot material for better water use efficiency in tomato plants

H Kurnia, R R Rifadi, Agustono, M N G Amin, S A Sudjarwo and M A Alamsjah

+ Open abstract View article PDF

OPEN ACCESS 012111

Characterization of edible coating based on surimi fillet catfish as biodegradable packaging

E Saputra

+ Open abstract View article PDF

OPEN ACCESS 012112

Characteristics of the fish protein isolate recovered from Sardine by-products using the Isoelectric Solubilization- Precipitation method

H. Pramono, N T Irawan and M R A Firdaus

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DPPH scavenging property of bioactives from soft corals origin palu bay, Central Sulawesi, Indonesia

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Extraction of bioactive compounds fruit from *Rhizophora mucronata* using sonication method

Ernawati, E Suprayitno, Hardoko and U Yanuhar

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Harvesting *Chaetoceros gracilis* by flocculation using Chitosan

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The Potential of Lindur Fruit Flour (*Bruguiera Gymnorrhiza*) in Reducing Oil Absorption of Milkfish Nugget during the Deep Frying Process

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R Ningsih, Sudarno and Agustono

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The Increase in β-carotene Content in *Dunaliella salina* from the Application of Different Light Intensities

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The Increase in β-carotene Content in *Dunaliella salina* from the Application of Different Light Intensities

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Abstract. *Dunaliella salina* is an example of a natural source of β-carotene and it is made up of larger cells than others in the genus *Dunaliella*. It is therefore able to produce more β-carotene. *Dunaliella salina* is able to accumulate more β-carotene content when cultured in environmental stress conditions such as high salinity, low nutrients and high light. This study aims to determine the increase in β-carotene content in *D. salina* due to the application of difference light intensities and to further determine the maximal light intensity required to obtain the highest β-carotene content in *D. salina*. The treatments used were different light intensities of700 Lux, 2.200 Lux, 3.700 Lux and 5.200 Lux with five repetitions respectively. The primary parameter that was measured was the β-carotene content of *D. salina*. The supporting factors in this research were the growth of *D. salina* and the water quality; i.e. temperature, pH, and salinity. The results showed that different light intensities can increase the β-carotene content in *D. salina* with the increased light intensity being the treatment. The maximum light intensity required to produce the highest amount of β-carotene content was 5.200 Lux with 0,0087 ml/L. Based on these results, further research is needed regarding light intensities to see if it can tolerate further stimulated growth in order to produce β-carotene more.

1. Introduction

Photosynthetic organisms have organic pigments that play a role in the process of photosynthesis such as chlorophyll, carotenoids and phycobilin. Carotenoids are commonly found in plants, algae, photosynthetic bacteria, non-photosynthetic bacteria, fungi and molds [1]. More than 400 carotenoids are found in nature and β-carotene is the most widely commercialized carotene [2]. There are two kinds of carotenoids used in aquaculture, natural and synthetic, which when used excessively can cause damage to the environment and the price of synthetic β-carotene is also more expensive than natural β-carotene [3].

β-carotene is a hydrocarbon ($C_{40}H_{56}$) with saturated chains that produce the color orange. It has two isomers, namely trans and cis. These molecules act physiologically as pro-vitamin A and free-radical scavengers [4]. β-carotene is widely used as an anti-cancer treatment, and it also prevents premature aging with the immunomodulators serving as a coloring additive in aquaculture and as an antioxidant [2]. Dunaliella has several advantages compared to natural sources of β-carotene. The advantages include continuous cultivation being easier due to the shorter culture time and a higher rate of cell growth. Dunaliella is easily cultivated because it is more resistant to changing environmental conditions.

A local isolate algae species of Brackish Water Aquaculture Institute (BBAP) Jepara, *D. salina* is a potential source of carotenoids as a feed additive or as a feed supplement in fish farming [5]. *Dunaliellasalina* is an unicellular green alga, which can grow in a variety of environmental conditions such as a broad range of low temperature, salinity, pH, and high light [6]. *Dunaliellasalina* can accumulate more of a concentration of β-carotene when cultured under conditions of environmental stress. The intensity of the light will affect the cellular responses of *D. salina;* the cells will release chlorophyll and other pigments will increase as phycobiliprotein and β-carotene are a form of self-defense [7]. The results of the study [8] showed that when cultured in salinity stress conditions and low nitrogen levels, *D. salina* is capable of producing β-carotene, which makes up 60.4% of the total carotenoids. Based on the above, we have conducted research to determine if the increase in the content of β-carotene can be due to different light intensities. This study aims to determine the increase in the content of β-carotene in *D. salina* when due to different light intensities and to determine the best light intensity that can produce the highest content of β-carotene in *D. salina*.

2. Materials And methods

2.1 Materials

The tools used in this study were a glass tube, aerator, test tubes, test tube rack, measuring cup, pipette, micro-pipette, binocular microscope, haemocytometer, hand counters, spectrophotometers, fluorescent light 40-watt, rack culture, refractometer, pH meter, lux meter, autoclave, cover glass, paper label, vortex, centrifuge, cuvettes, dark plastic, styrofoam and aluminum foil. The materials used in this study were *D. salina* seeds and medium Walne derived from the Brackish Water Aquaculture Institute (BBAP) Situbondo, sea water, methanol, acetone, alcohol, soap, wipes and distilled water.

2.2 Method

The research activities were carried out at the Laboratory of Education in the Faculty of Fisheries and Marine Resources of Universitas Airlangga in Surabaya. The method used in this study was an experimental method using a completely randomized design (CRD) with four treatments and five replications. The treatments were the difference in the intensity of light on the culture of D. salina. The intensities of the light given were 700 Lux (A), 2200 Lux (B), 3,700 Lux (C) and 5.200 Lux (D) respectively.

2.2.1 Preparation and light intensity settings

The culture medium used was medium Walne obtained from the Brackish Water Aquaculture Institute (BBAP) Situbondo. There were 20 experimental units, which used 20 glass tubes. The prepared culture space was created by installing a shelf culture and every part of the sides was covered with dark plastic. The lighting treatment was created using 40-Watt fluorescent lamps placed on racks. The values obtained determined the light intensity on through the distance between the lamp and a bottle of the culture media. The measurement of light intensity was done using a Lux meter [9]. The light intensity measurements were conducted using a Lux meter, exposing the sensor lens on the lamp to the light until the needle on the scale showed the desired number.

2.2.2 Sterilization

Sterilization included the sterilization of the equipment, materials and culture rack. The equipment was washed with fresh water and dried in the sun. The equipment used for culturing was sterilized using an autoclave at 121 ℃ for 15 minutes. Sterilization using an autoclave was also done for the equipment made of heat-resistant glass. The media sterilization was done by inserting the media into sea water in a glass bottle and then covering it using coated cotton gauze and aluminum foil [10]. Glass bottles containing the medium were sterilized using an autoclave at a temperature of 121ºC for 15 minutes.

2.2.3 Fertilizer preparation for culture D. salina

Fertilizer was used as a culture medium in the laboratory; we used Walne fertilizer obtained from Brackish Water Aquaculture Institute (BBAP) Situbondo. The composition of the fertilizer was Na_2EDTA 45 g, $NaH_2PO_4.H_2O$ 20 g, $FeCl_3.6H_2O$ 1.5 g, H_3BO_3 33.6 g, $MnCl_2$ 0.36 g, $NaNO_3$ 100 g trace metal solution 1 ml, vitamin 1 ml and 100 ml of distilled water. The fertilizer solution was sterilized using an autoclave and then stored in the refrigerator [10].

2.2.4 Environment and culture media - D. salina

The environment and culture can affect the growth of *D. salina*, therefore the same conditioned environment was needed for each treatment. The culture media used in this study was sterile sea water with a salinity of 30 ppt. The volume of the total culture of *D. salina* was 500 ml, and was a mixture of 464 ml of sterile sea water, *D. Salina* seeds 35 ml with a pure stock density of 719.825 x 10^4 cells / ml and with the desired density of 5x10^5 cells / ml, fertilizer from Walne by 0.5 ml and 0.5 ml of vitamin B12 [11].

The D. salina seeds used for the study were obtained from the Brackish Water Aquaculture Institute (BBAP) Situbondo. The delivery of the seeds of *D. salina* was carried out at low temperatures. The seed were packaged into a bottle and then put in a Styrofoam box that had been filled with ice, and then the seed were stored in the refrigerator. The seedsof pure stock were checked and counted using a haemocytometer under the microscope before inserting them into the culture bottle. The seeds were put in a culture flask at a density of 5x10^5 cells / ml. The numbers of seeds necessary for culturing were calculated using the following formula [12]:

$$V1 = \frac{N2XV2}{N1}$$

Information:

V1 = Volume of *Dunaliella salina* required (ml)
V2 = Volume of the desired culture media (ml)
N1 = Density of pure seeds or stock of *Dunaliella salina* (cells / ml)
N2 = Density of the seedlings of the *Dunaliella salina* desired (cells / ml)

2.2.5 Measurement growth of D. salina

The growth measurements were performed by calculating the density of the cells every day until harvesting. The sampling was done using a pipette. The cell density was calculated by direct counting using a haemocytometer and then counting the cells under a dissecting light microscope with a magnification of 400 times. The data obtained was calculated using the following formula[12]:

Phytoplankton Density (cell/ml) = $\frac{nA+nB+nC+nD}{4}$ x 10^4

Note: nA, nB, nC, nD = Number of phytoplankton in block A, B, C, D

2.2.6 Harvesting D. salina

Phytoplankton harvesting should be done at the right time, at the peak of the population [10]. Harvesting D. salina was done partially on the 2nd, 4th, 6th, 8th, 10th, 12th and 14th [2]. This method intended to find differences in the growth phase changes in each treatment and to better know the progress of the formation of β-carotene in *D. salina*. *D. salina* was partially harvested by taking a 10 ml sample, putting it into a test tube and then extracting it to determine the content of β-carotene in *D. salina* [10].

2.2.7 Extraction

The analysis of the content of β-carotene was done by taking a 5 ml sample yield of *D. salina* using a pipette, and then centrifuging it at a speed of 5,000 rpm for five minutes [2]. The results of the centrifuge were then taken and added to 5 ml of distilled water and centrifuged at a speed of 5,000 rpm for five minutes. The results were then made into a pellet and added to 5 ml acetone: distilled in water at a ratio (80:20 v/v) of 4 ml acetone and 1 ml of distilled water. The resulting mixture was homogenized using a vortex for two minutes and centrifuged at a speed of 5,000 rpm for five minutes. The resulting mixture was composed of two layers of liquid fluid; the colors were clear and yellowish liquid respectively. The layers of clear liquid and yellowish liquid were taken to be read on a spectrophotometer with a wavelength of 480 nm.

2.2.8 Calculation the content of β-carotene

The extraction of the coating of yellowish liquid was then inserted into the corresponding cuvettes and read using a spectrophotometer with a wavelength of 480 nm [2]. The B-carotene calculation formula was as follows [13]:

$$\text{Content of β-karoten (ml/L)} = \frac{A \; x \; V \; x \; 1000}{A_{1cm}^{1\%} \; x \; 100}$$

Note:

$A_{1cm}^{1\%}$: coefficient of the absorbance of β-carotene 2273
V : sample volume
A : absorbance at a wavelength of 480 nm

3. Results and discussion

3.1 Result

The B-carotene content of *D. salina* was measured every other day, starting on day 2 through to day 14 in order to determine the development of the formation of β-carotene in *D. salina*. The data on the average β-carotene content of *D. salina* in all treatments has been presented in Table 1.

Table 1. Content of β-carotene in *D. salina*

Day	Content of β-carotene in *D. salina* (ml/L) ± SD			
-	A (700 Lux)	B (2.200 Lux)	C (3.700 Lux)	D (5.200 Lux)
2	$0{,}0012^{c}$± 0,0002	$0{,}0014^{c}$± 0,0003	$0{,}0032^{b}$± 0,0010	$0{,}0054^{a}$± 0,0013
4	$0{,}0024^{b}$± 0,0010	$0{,}0043^{ab}$± 0,0009	$0{,}0054^{a}$± 0,0021	$0{,}0058^{a}$± 0,0027
6	$0{,}0031^{c}$± 0,0008	$0{,}0049^{b}$± 0,0012	$0{,}0059^{ab}$± 0,0015	$0{,}0074^{a}$± 0,0012
8	$0{,}0035^{c}$± 0,0015	$0{,}0052^{bc}$± 0,0052	$0{,}0068^{ab}$± 0,0011	$0{,}0087^{a}$± 0,0014
10	$0{,}0052^{a}$± 0,0011	$0{,}0053^{a}$± 0,0008	$0{,}0051^{a}$± 0,0007	$0{,}005^{a}$7±0,0010
12	$0{,}0042^{a}$± 0,0019	$0{,}0043^{a}$± 0,0013	$0{,}0043^{a}$± 0,0005	$0{,}0054^{a}$± 0,0008
14	$0{,}0037^{a}$± 0,0012	$0{,}0039^{a}$± 0,0021	$0{,}0036^{a}$± 0,0016	$0{,}0041^{a}$± 0,0022

Note: Different superscript letter notations in the same column show the comparison between treatments and that there is a very significant difference ($P < 0.05$).

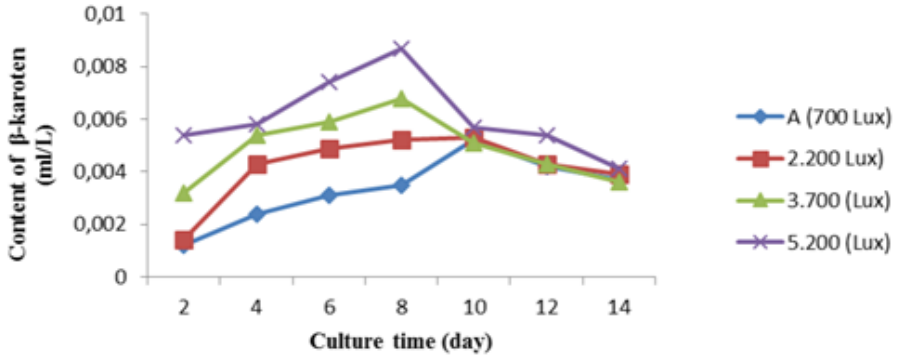

Figure 1. β-carotene content from *D. salina*

Based on Table 1 and Figure 1, the highest content of β-carotene was in treatment A (light intensity of 700 Lux) and B (light intensity 2,200 Lux), which occurred on the 10th day in 0.0052 ml / L and in 0.0053 ml / L. The C treatment (light intensity 3,700 Lux) and D treatment (the light intensity of 5,200 Lux) in relation to the β-carotene content highs occurred on the 8th day at 0.0068 ml / L and 0.0087 ml / L respectively.

D. salina growth can be illustrated with the cell densities calculated every day for 14 days of maintenance by using a haemocytometer. The data and graph density showing the *D. salina* growth can be seen in Figure 2.

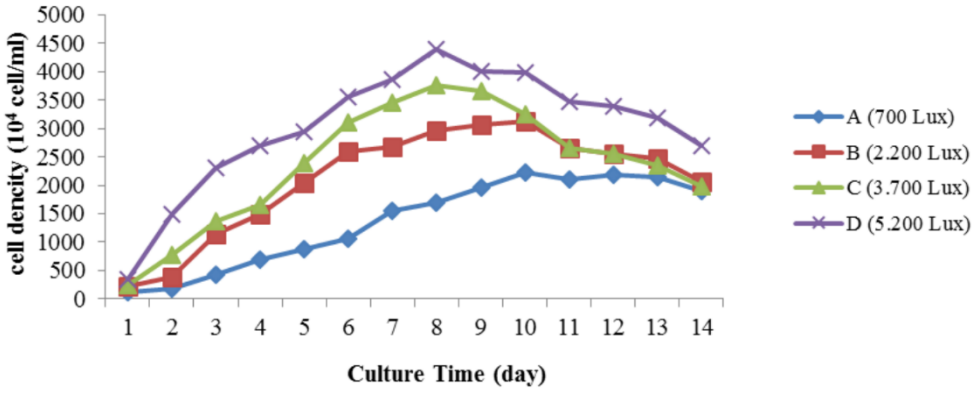

Figure 2. Density of *D. salina*

Based on Figure 2, it can be seen that the density of *D. salina* follows the pattern of growth of phytoplankton culture in general; the adaptation phase, exponential growth, stationary period and death. In treatment A and B (light intensity of 700 Lux, and 2200 Lux), the 1st to the 2nd day was the adaptation phase, the exponential phase occurred on the 3rd day until the 10th day and the death phase started on the 11th day until the end of the culture on the 14th day. The density of *D. salina* in

treatments C and D (light intensity 3,700 and 5,200 Lux) showed that the 1st and 2nd days were the adaptation phase, the exponential phase occurred on day 3 to day 8and the growth started to decrease in density (death phase) from day 9th until the end of the 14th day of culture.

3.2 Discussion

The results of the calculation of the β-carotene content of D. salina on the 2nd day through to the 14th showed that the β-carotene content of D. salina was the highest in treatment D (the light intensity of 5,200 Lux). This is presumably as the high light causes the enzyme in the cells of *D. salina* to work optimally for the formation of β-carotene. The high intensity light is capable of increasing the carotenoid hydroxylase enzyme (CH) and phytoene synthase (PSY), which is a precursor of phytoene. The increased carotenoid hydroxylase enzyme (CH) and phytoene synthase (PSY) causes the amount of phytoene to also increased [14]. The phytoene constituent component is β-carotene, thus the synthesis of β-carotene also increased.

β-carotene can be formed at the beginning of the growth stage along with chlorophyll-a, which serves as a photoprotector that prevents damage from photooxidation in the process of chlorophyll release. This is as it is easily damaged because the chlorophyll will form a triplet which binds with oxygen to form a singlet oxygen (singlet oxygen) [15]. Singlet oxygen is an oxidant that will oxidize the chlorophyll, fatty acids, proteins and nucleic acids, leading to the death of the organism. β-carotene will immediately neutralize the singlet oxygen through a detoxification process so then cell death does not occur. If the environmental conditions are not appropriate then it will lead to the physiological conditions of the *D. salina* cells not being balanced, so then the synthesis of β-carotene increased as a form of self-defense [2]. The production of β-carotene in the *D. salina* cells serves as an antidote to thefree radicals and harmful toxins that enter the cell.

On the 10th, 12th and 14th days, the average β-carotene content of D. salina showed similar results between the treatments. The average value of the content of β-carotene, which is almost the same on the 12th day and the 14th, is suspected to be caused by the cells in *D. salina* for all treatments when it is going through the death phase (Table 2). This causes the content of the β-carotene produced to also decrease. The production of the carotenoid pigments in *D. salina* are increasing in line with the increasing age of the cells; this will continue to decline until the cell death phase [15]. The production of β-carotene in this phase is used for cell survival when the nutrients in the medium begin to thin.

Light plays an important role in the formation of β-carotene in *D. salina* cells. The *D. salina* cells will respond to light, and then the cells will activate signal transduction from the cell nucleus to the plastid [16]. The activation of the signal transduction will cause a change in the gene expression in the nucleus and cytoplasm. The products of these genes will be sent to the chloroplast, and then the formation of β-carotene will begin, prompted by enzyme karotenogenesis. The carotenoid biosynthetic process begins with two molecules of GGPP (C20) that condense into phytoene due to the enzyme phytoene synthase (PSY) [17]. The biosynthesis of phytoene desaturation is followed by a reaction due to the enzyme phytoene desaturase (PDS) to produce the trans lycopene which is pink and 9,15,9'-tri-cis-ζ-carotene. This reaction is catalyzed by two desaturase and two isomerase. The first desaturase produces 9,9'-di-cis-ζ-carotene by the enzyme carotene isomerase (Z-ISO). The second desaturase produces 7,9,9'-cis-neurosporene and 7 ', 9'-cis-lycopene by the enzyme desaturase (ZDS). The carotene isomerase enzyme (CRTISO) will catalyze the formation of all-trans-lycopenes. Lycopene cyclase will undergo two reactions, namely by the enzyme β-cyclase (βLCY) to produce β-carotene and ε-cyclase enzyme (εLCY) which can produce α-carotene.

Phytoplankton growth consists of four phases; adaptation, exponential, stationary and death [10]. The adaptation phase in all treatment occured on day 1 until the 2nd day; i.e. cell growth had not occurred significantly due to *D. salina* still adapting to the environment. The exponential phase of treatments A and B (light intensity of 700 Lux, and 2200 Lux respectively) occurred on day 3 to day 10 in treatments C (the light intensity of 3,700 Lux) and D (the light intensity of 5,200 Lux) until the 8th day. The stationary phase in all of the treatments did not appear, presumably because the stationary

phase lasts less than 24 hours while the observation cell density of *D. salina* was done once every 24 hours.

The death phase in treatment A (light intensity of 3,700 Lux) and B (light intensity 2,200 Lux) started on the 11th day, while in treatment C (light intensity of 3,700 Lux) and D (light intensity of 5,200 Lux), it started on the 9th day. This happened because in treatment C (light intensity of 3,700 Lux) and D (the light intensity of 5,200 Lux), there was more rapid cell division so then the number of cells increased. The higher the number of cells in the culture medium, the more nutrients are needed for growth. This resulted in a nutrient culture medium that decreased, so then in treatment C (light intensity of 3,700 Lux) and D (light intensity of 5,200 Lux), the phase of early death occurred. The quality of the water and nutrients in the media can lead to diminishing phytoplankton cells that cannot thrive, causing cell death [18]. Phytoplankton will utilize the nutrients in the culture medium until the peak cell densities require more nutrients, while the nutrient media will decline because there is no addition of nutrients [19]. This can cause cell death in the phytoplankton.

D. salina can reproduce in two ways: sexual and asexual [5]. Sexual reproduction can occur in response to environmental changes that are not appropriate, through the process of gametogenesis by producing isogametes that looks like zoospores [16]. *D. salina's* sexual reproduction occurs by way of isogamy, as zygote developments will occur in meiotic division [20]. Red or green zygotes have an endomembrane that is smooth and very thin. The zygote will divide in meiosis after the resting phase and will be comprised of more than 16 cells released through a crack in the wall of the stem cells. This will split into 32 haploid daughter cells [16].

Changes in salinity are suspected of being the cause of stress in the *D. salina* cells in addition to the treatment of light intensity. Changes in salinity can damage the cell's plasma membrane, thus triggering the activation of the enzyme protein kinase that may trigger the conversion of starch into glycerol in the chloroplasts [21]. Salinity changes that take place over a long period will cause the enzyme protein kinase to work to change gene expression through the transcription process so as to produce carotenoids.

4. Conclusion

Different light intensity affects the β-carotene content of *D. salina*. The higher the light intensity that is given, the higher the β-carotene content that is produced. The optimal light intensity needed to produce the highest β-carotene content is 5200 Lux paired with 0,0087 ml/L.

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