LAPORAN AKHIR

PENELITIAN STRATEGIS NASIONAL

INSTITUSI

ISOLASI DAN SKRINING OLEAGINOUS MIKROALGA LAUT DI PERAIRAN KENDARI YANG POTENTIAL UNTUK DIKULTUR MASSAL PADA HYPERSALINE MEDIA SEBAGAI BIODIESEL FEEDSTOCK

Tahun ke 2 dari rencana 3 tahun

Ketua/Anggota Tim

INDRAYANI, S.Pi, M.Biotech.Stu.Ph.D (NIDN: 0023127404)

HASLIANTI, S.Pi, M.Si (NIDN: 0017077906)

Dr. ASRIYANA, S.Pi, M.Si (NIDN: 0011127604)

UNIVERSITAS HALUOLEO

NOVEMBER 2018

HALAMAN PENGESAHAN

Judul	: ISOLASI DAN SKRINING OLEAGINOUS MIKROALGA LAUT DI PERAIRAN KENDARI YANG POTENTIAL UNTUK DIKULTUR MASSAL PADA HYPERSALINE MEDIA SEBAGAI BIODIESEL FEEDSTOCK
Peneliti/Pelaksana	
Nama Lengkap	: INDRAYANI, S.Pi, M.Biotech, Ph.D
Perguruan Tinggi	: Universitas Halu Oleo
NIDN	: 0023127404
Jabatan Fungsional	: Lektor
Program Studi	: *Manajemen Sumber Daya Perairan*
Nomor HP	: 082188629424
Alamat surel (e-mail)	: indrayani_tajudin@yahoo.com.au
Anggota (1)	
Nama Lengkap	: HASLIANTI S.Pi, M.Si
NIDN	: 0017077906
Perguruan Tinggi	: Universitas Halu Oleo
Anggota (2)	
Nama Lengkap	: Dr ASRIYANA S.Pi. M.Si
NIDN	: 0011127604
Perguruan Tinggi	: Universitas Halu Oleo
Institusi Mitra (jika ada)	
Nama Institusi Mitra	:
Alamat	:
Penanggung Jawab	:
Tahun Pelaksanaan	: Tahun ke 2 dari rencana 3 tahun
Biaya Tahun Berjalan	: Rp 140,000,000
Biaya Keseluruhan	: Rp 395,727,000

Mengetahui,
Dekan FPIK UHO

(Prof. Ir. H. La Sara, M.Si, Ph.D)
NIP/NIK 196004221897031003

Kota Kendari, 14 - 11 - 2018
Ketua,

(INDRAYANI, S.Pi, M.Biotech, Ph.D)
NIP/NIK 132297346

Menyetujui,
Ketua LPPM UHO

(Dr. H. La Aba, S.Si, M.Si)
NIP/NIK 196912311997031011

LAPORAN AKHIR

PENELITIAN STRATEGIS NASIONAL

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Ketua/Anggota Tim

INDRAYANI, S.Pi, M.Biotech.Stu.Ph.D (NIDN: 0023127404)

HASLIANTI, S.Pi, M.Si (NIDN: 0017077906)

Dr. ASRIYANA, S.Pi, M.Si (NIDN: 0011127604)

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Peneliti/Pelaksana	
Nama Lengkap	: INDRAYANI, S.Pi, M.Biotech, Ph.D
Perguruan Tinggi	: Universitas Halu Oleo
NIDN	: 0023127404
Jabatan Fungsional	: Lektor
Program Studi	: Manajemen Sumber Daya Perairan
Nomor HP	: 082188629424
Alamat surel (e-mail)	: indrayani_tajudin@yahoo.com.au
Anggota (1)	
Nama Lengkap	: HASLIANTI S.Pi, M.Si
NIDN	: 0017077906
Perguruan Tinggi	: Universitas Halu Oleo
Anggota (2)	
Nama Lengkap	: Dr ASRIYANA S.Pi, M.Si
NIDN	: 0011127604
Perguruan Tinggi	: Universitas Halu Oleo
Institusi Mitra (jika ada)	
Nama Institusi Mitra	:
Alamat	:
Penanggung Jawab	:
Tahun Pelaksanaan	: Tahun ke 2 dari rencana 3 tahun
Biaya Tahun Berjalan	: Rp 140,000,000
Biaya Keseluruhan	: Rp 320,000,000

Mengetahui,
Ketua LPPM UHO

(Dr. H. La Aba, S.Si, M.Si)
NIP/NIK 19691231 1997031011

Kota Kendari, 28 - 8 - 2018
Ketua,

(INDRAYANI, S.Pi, M.Biotech, Ph.D)
NIP/NIK 132297346

RINGKASAN

Kebutuhan dunia akan bahan bakar fosil yang terus meningkat sementara persediaan yang semakin menipis ditambah lagi dengan masalah lingkungan yang ditimbulkan oleh penggunakan fosil fuel yaitu Green House Gases (GHG) telah memotivasi para scientists untuk memikirkan berbagai alternative sumber biofuels terutama biodiesel.Mikroalga sebagai alternative feedstock untuk produksi biodiesel telah menjadi perhatian secara global sejak beberapa tahun terakhir dikarenakan mikroalgae memiliki banyak keunggulan dibandingkan biodiesel feedstock lainnya seperti canola, rapeseed, jatropha, coconut maupun palm oil. Mikroalgae memiliki pertumbuhan yang cepat (dapat di panen setiap hari), kandungan lipid yang tinggi (oleaginous microalgae) hingga 80% dari berat kering biomass yang dapat dikonversi menjadi biodiesel sertalebih sustainable karena dapat dikultur pada lahan yang tidak produktif untuk tanaman pangan, dapat memanfaatkan air laut serta tidak akan memicu issue "food vs fuel feud".Selain itu, mikroalga memiliki banyak senyawa lain yang dapat menjadi produk sampingan dari biodiesel. Kultur mikroalga juga dapat diintegrasikan dengan pengolahan limbah cair maupun untuk CO_2 bioremediation.

Meskipun memiliki banyak kelebihan namun banyak juga permasalahan yang menghambat komersialisasi microalgae sebagai biodiesel feedstock. Permasalahan terbesar adalah tingginya biaya produksi. Salah satu upaya yang dapat dilakukan untuk mengurangi tingginya biaya produksi adalah seleksi species/strainyang sangat productive yang dapat dikultur sepanjang tahun pada kondisi iklim yang optimal dengan menggunakan kultur sistem yang biaya kapital dan operasionalnya lebih murah yakni sistem kolam terbuka (open pond sistem). Mikroalga laut yang dapat tumbuh dengan baik pada salinitas yang tinggi (hypersaline) adalah yang paling potensial dan lebih sustnaible sebagai biodiesel feedstock karena lebih memungkinkan untuk dikembangkan secara massal dengan sistem kolam terbuka dengan memanfaatkan air laut dan lahan-lahan yang tidak produktif untuk tanaman pertanian.Selain itu,kultur massal pada hypersaline media akan mengurangi resiko kontaminasi oleh non-target spesies yang merupakan permasalah utama pada kultur massal outdoor dan menjadi penyebab umum kultur kolaps.

Penelitian ini bertujuan untuk mengisolasi species lokal microalgae laut yang memiliki kandungan lipid yang tinggi, pertumbuhan yang cepat serta dapat dikultur secara massal di outdoor menggunakan media dengan salinitas tinggi (≥ 3% NaCl) untuk produksi biodiesel. Penelitian ini direncanakan dalam 3 tahun. Tahun ke-1 melakukan isolasi species lokal mikroalga laut dari berbagai habitat perairan asin (estuaria dan pantai/ laut) perairan sekitar kota Kendari, Sulawesi Tenggara (pantai Nambo, pantai Toronipa, pulau Bokori, muara sunagn Wanggu dan Lasolo). Tahun ke-2 melakukan skrining/seleksi terhadap isolate microalgae yang memiliki karakeiristik unggul yakni pertumbuhan yang cepat, kandungan lemak yang tinggi serta kemampuannya untuk hidup pada salinitas yang tinggi (>3% NaCl) dan tahun ke-3 melakukan ujicoba kultur massal di outdoor open pond system (raceway ponds) terhadap species-species yang unggul (2-3 speceis unggul).

Target luaran dari penelitian ini adalah 1). Akan diperoleh puluhan hingga ratusan isolate species lokal mikroalga laut. 2). Rekomendasi species/strain yang paling potensil sebagai biodiesel feedstock. 3). Akan dihasilkan beberapa publikasi baik pada jurnal nasional maupun jurnal international. 4). Hasil penelitian juga akan dipresentasikan pada konferensi Nasional dan International. 5). Dapat memperkaya bahan ajar mata kuliah planktonologi, budidaya pakan alami, bioteknologi perairan dan bioprospecting kelautan.

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BAB I. PENDAHULUAN

Mikroalgae adalah mikroorganisme prokaryotic maupun eukaryotic yang berphotosynthesis yang dapat ditemukan pada hampir semua ekosistem baik aquatic maupun terestrial (Richmond 2004; Mata et al. 2010). Mikroalgae merupakan salah satu sumber biodiesel yang paling menjanjikan untuk dikembangkan sebagai alternative dari bahan bakar fosil untuk memenuhi kebutuhan global akan bahan bakar (Chisti 2007). Upaya pengembangan microalgae sebagai sumber biodiesel sedang dilakukan secara extensive di seluruh dunia (Wijffels and Barba 2010). Beberapa keunggulan yang dimiliki oleh mikroalga sebagai biodisesl feedstock diantaranya kandungan lipid/oil microalgae yang sangat tinggi (Schenk et al. 2008). Pertumbuhan yang cepat dan bisa menggandakan biomas dalam hitungan jam (Spolaore et al. 2006; Chisti 2007). Produksi mikroalga tidak mengganggu rantai suplay makanan dibandingkan dengan sumber biodiesel lainnya seperti kelapa sawit, jagung dan kedelai (Chisti 2007). Produksi microalgae tidak berkompetisi lahan dengan produksi tanaman pangan dikarenakan produksi mikrolaga tidak membutuhkan lahan yang sangat luas serta dapat memanfaatkan lahan tidur yang tidak dapat dimanfaatkan untuk produksi tanaman pangan seperti lahan kering dan lahan yang terpengaruh air laut sehingga lebih sustainable (Borowitzka and Moheimani 2013b). Mikroalgae mengandung senyawa/bahan kimia lainnya yang dapat dihasilkan sebagai produk sampingan seperti pigment bernilai tinggi, protein dan carbohydrate serta residue biomass yang dapat dimanfaatkan sebagai pakan atau fertiliser ataupun untuk mengasilkan ethanol dan methane(Spolaore et al. 2006). Microalgae memiliki kemampuan untuk memanfaatkan CO_2 dari hasil buangan industry (flue gasses) sehinggan dapat diusahakan secara untuk produksi biomass dan bioremediasi CO_2 serta dapat juga dimanfaatkan untuk pengolahan limbah cair dikarenakan microalgae dapat memanfaatkan nutrient yang berlebihan pada limbah cair ataupun mengabsorbsi limbah logam berat sehingga dapat diusahakan secara bersama-sama untuk pengolahan limbah cair dan produksi biomass mikroalga (Chisti 2007).

Pengembangan microalgae sebagai biodiesel feedstock di Indonesia sangat jauh tertinggal dibandingkan negara-negara lain seperti Australia, Amerika, Kanada, Jerman dan China. Padahal Indonesia memiliki potensi yang jauh lebih besar untuk memproduksi biodiesel dari microalgae. Indonesia adalah negara tropis yang memiliki iklim yang sesuai untuk membudidayakan mikroalga (suhu dan cahaya matahari yang optimal sepanjang tahun) sehingga produktivitas akan tinggi sepanjang tahun. Indonesia memiliki ribuan pulau sehingga memiliki daerah pesisir/pantai yang sangat banyak dan luas yang merupakan lokasi ideal untuk kultur microalgae laut. Indonesia memiliki biodiversity microalgae yang sangat besar yang belum banyak dieksplorasi. Menurut Borowitzka (personal communication), pengembangan microalgae untuk biodiesel lebih dimungkinkan di negara-negara dimana iklimnya mendukung pertumbuhan mikroalga sepanjang tahun, lahan dan tenaga kerja tersedia dengan biaya yang lebih murah sehingga biaya produksi akan jauh lebih rendah. Penelitian tentang mikroalga untuk biodiesel sudah banyak dilakukan di Indonesia namun belum ada yang secara specific memfokuskan penelitian pada microalgae yang mampu hidup pada lingkungan yang hypersaline padahal ini merupakan salah satu syarat penting untuk keberhasilan produksi massal mikroalga menggunakan sistem kultur kolam terbuka di outdoor (outdoor open pond systems) terutama untuk produksi biodiesel.

Kultur massal microalgae untuk biofuels membutuhkan air yang sangat banyak (Borowitzka and Moheimani 2013b; Fon Sing et al. 2013). Jika menggunakan air tawar maka akan secara langsung berkompetisi dengan tanaman pangan, industry dan rumah tangga untuk penggunaan sumber daya air tawar yang terbatas. Sebaliknya penggunaan air laut/asin lebih sustainable dan ekonomis (Yang et al. 2011; Resurreccion et al. 2012). Namun, mikroalga yang dikultur dengan menggunakan media air laut khususnya pada kolam terbuka akan mengalami fluktuasi salinitas disebakan oleh evaporasi dan hujan dan salinitas medium akan sangat tergantung apakah air tawar atau air laut yang digunakan untuk menggantikan

kehilangan air akibat evaporasi. Jika air tawar yang digunakan untuk menggantikan kehilangan air akibat evaporasi maka akan sangat banyak air tawar yang dibutuhkan sementara jika air laut yang digunakan maka salinitas medium akan terus meningkat. Oleh karena itu untuk keberhasilan dan sustainabilitas dari kultur massal jangka panjang menggunakan air laut, maka mikroalga yang memiliki toleransi yang luas terhadap salinitas menjadi sangat penting (Borowitzka and Moheimani 2013b). Selain itu mikroalga yang dikultur pada hypersaline media tidak akan mudah terkontaminasi oleh organisme lain termasuk protozoa, zooplankton maupun jenis microalgae lainnya sehingga akan lebih memungkinkan untuk di kultur secara massal di outdoor untuk jangka panjang (Borowitzka 2013b). Untuk produksi biodiesel, spesies/strain yang euryhaline pada lingkungan yang hypersaline saja tidak cukup. Strain yang unggul harus juga memiliki karaketristik lainnya yaitu kandungan lipid yang tinggi, pertumbuhan yang cepat sehingga produkstifitas biomass dan lipidnya juga akan tinggi. Hal inilah yang menjadi alasan pentingnya untuk terus melakukan skrining untuk mendapat strain yang terbaik untuk meningkatkan produktivitas biomass dan lipid (Barclay and Apt 2013).

BAB 2. TINJAUAN PUSTAKA

Microalgae merupakan feedstock yang paling potensial untuk produksi biofuels terutama biodiesel(Ndimba et al. 2013). Biodiesel dari microalgae telah direview secara ekstensive oleh beberapa penulis (Chisti 2007; Hu et al. 2008; Mata et al. 2010). Potensi microalgae sebagai biodiesel feedstock adalah karena kemampuannya untuk mengakumulasi lipid dalam jumlah yang banyak yang dapat dikonversi menjadi biodiesel (Parmar et al. 2011). Mikroalgae untuk produksi biodiesel lebih sustainable karena dapat dikultur pada lahan yang tidak produktif untuk tanaman pertanian (non-arable land) dan dapat memanfaatkan air laut sehingga tidak akan berkompetisi dengan tanaman pangan untuk lahan maupun air tawar (Borowitzka and Moheimani 2013b). Mikroalga juga dapat memanfaatkan buangan gas dari industry sebagai sumber karbon (Chisti 2007)

Lipid yield dari mikroalga jauh lebih tinggi dibandingkan tanaman darat. Mikroalga dapat menghasilkan 58.700 – 136.900 L oil $ha^{-1}year^{-1}$dibandingkan dengan soybean (636 L oil $ha^{-1}year^{-1}$), jatropha (741 L oil $ha^{-1}year^{-1}$), canola (974 L oil $ha^{-1}year^{-1}$) dan palm oil (5366 L oil $ha^{-1}year^{-1}$) (Ahmad et al. 2011). Kandungan lipid mikroalga sangat bervariasi tergantung spesies/strain. Kandungan lipid dari ratusan mikroalga yang telah diteliti berkisar antara 1 – 85% dari berat kering biomass(Spolaore et al. 2006; Chisti 2007). *Nannochloropsis* spp dan *Botryococcus braunii* dapat mengakumulasi hingga 80% of lipid (Larkum et al. 2012)sementara*Chlorella pyrenoidosa* dapat mengakumulasi hingga 85% lipid dalam kondisi kekurangan nitrogen(Rodolfi et al. 2009).

Beberapa faktor penting yang perlu dipertimbangkan dalam pengembangan biodiesel dari mikroalga. Seleksi spesies/strain adalah faktor yang pertama dan terpenting yang menentukan keberhasilan komersialisasi mikroalga dan seleksi spesies yang tepat merupakan faktor terpenting untuk kesuksesan produksi biodiesel dari mikroalga (Borowitzka 2013b). Strain yang ideal untuk produksi biofuels harus memiliki karakeristik sebagai berikut: (1) mempunyai produktivitas lipid yang tinggi; (2) memiliki tolreansi yang luas terhadap suhu dan salinitas; (3) mikroalga laut (lebih baik yang hypersaline spesies) sehingga memungkinkan untuk dikultur massal di sistem kultur kolam terbuka (open pond sistem); (4) dapat memanfaatkan kelebihan nutrient pada limbah cair sehingga dapat diintegrasikan dengan sistem pengolahan limbah; (5) menghasilkan co-product yang bernilai tinggi; (6) serta dapat memanfaatkan buangan gas CO_2 dari industri(Sheehan et al. 1998; Borowitzka 2013b).Sampai saat ini sangat sulit untuk mendapatkan spesies yang bisa memenuhi semua kriteria tersebut diatas. Namun di yakini bahwa spesies yang memiliki adaptasi spesifik terhadap suatu lingkungan tertentu menjadi kunci keberhasilan produksi mikroalga secara komersial karena memungkinkan mikroalga terekspose pada kondisi lingkungan tertentu dimana tidak banyak organisme yang bisa bertahansetempat jika dibandingkan dengan menggunakan spesies yang diimport yang belum tentu sesuai dengan iklim/lingkungan setempat(Sheehan et al. 1998). Spesies-spesie microalgae yang telah diproduksi secara komersial memiliki lingkungan yang selektif termasuk diantaranya *Dunaliella salina* yang diproduksi secara komersil untuk produksi β-carotene (production plant terbesar di Australia) yang hidup pada salinitas yang sangat tinggi mencapai 30% NaCl, *Spirulina* yang menyukai lingkungan dengan pH tinggi (9-11) telah di produksi secara komersial oleh Dainippon Ink dan Chemicals di Hainan (China), Earthrise Nutritional farms di California dan Cyanotech di Hawaii (USA),*Chlorella* yang menyukai media dengan kandungan nutrient yang tinggi telah diproduksi secara komersial oleh Taiwan Chlorella Manufacturing and Co (Taiwan) dan juga di Klotze (Germany) dan *Haematococcus pluvialis* sebagai penghasil pigment astaxanthin telah dibudidayakan secara komersial di Hawaii, India dan Israel(Olaizola 2003; Pulz and Gross 2004). Faktor penting lainnya adalah lokasi kultur yang memungkinkan untuk produksi sepanjang tahun (high insulation and acceptable temperature range) (Brennan and Owende 2010; Fon Sing et al. 2013).

Produksi massal microalgae secara komersial dapat dilakukan melalui dua system yakni system terbuka (open system) atau system tertutup (photobioreactors)(Borowitzka 1999). Produksi mikroalga pada kolam terbuka telah digunakan sejak tahun 1950 an. Sistem terbuka dapat dikategorikan ke dalam: perairan alam (danau, laguna dan kolam) dan kolam buatan atau container. Raceway pond adalah system kultur terbuka di outdoor yang paling umum digunakan untuk produksi komersial mikroalga (Borowitzka and Moheimani 2013a).Raceway pond terdiri atas sebuah sirkuit dengan saluran yang parallel yang dilengkapi dengan paddlewheel yang digunakan untuk mensirkulasi kultur mikroalgae(Zittelli et al. 2013). Raceway pond dapat terbuat dari concrete atau galian ditanah yang kemudian dilapisi dengan plastic atau terbuat dari fibreglass. Sistem ini yang digunakan untuk produksi *Spirulina /Arthrospira* oleh Earthrise Nutritionals, LLC (California, USA) and Hainan DIC Microalgae (China) dan untuk memproduksi astaxanthin dari*Haematococcus pluvialis* oleh Cyanotech Co. (Hawaii, USA) dan Parry Agro Industries Ltd (India) (Zittelli et al. 2013)dan juga untuk produksi komersil *Dunaliella* (Borowitzka 2013a).

Sistem kultur lainnya adalah photobioreaktor dikembangkan dengan tujuan untuk menutupi kekurangan utama dari system kultur terbuka yakni masalah polusi dan kontaminasi (Tredici 2004). Yang termasuk system tertutup adalah tubular, plate, dan column photobioreactors. Photobioreaktor memungkinkan kultur tunggal spesies microalgae untuk jangka waktu yang lama karena kecil kemungkinan terjadi kontaminasi dan lebih cocok digunakan untuk spesies microalgae yang sensitive (Tredici and Materassi 1992; Tredici 2004, 2010). Namun, kapital cost dari system tertutup jauh lebih mahal dari pada system kolam terbuka. Dibandingkan dengan photobioreaktor, sistem kolam terbuka merupakan metode produksi massal biomas mikroalga yang paling murah dan lebih banyak digunakan untuk produksi komersil mikroalga (Borowitzka and Moheimani 2013a; Borowitzka and Moheimani 2013b).

Study tentang pengembangan mikroalga sebagai biodiesel feedstock sudah sangat banyak dilakukan namun kebanyakan masih terbatas pada skala kecil di laboratorium dan belum banyak yang sampai pada kultur massal terutama yang menggunakan sistem kolam terbuka serta secara spesifik fokus pada lingkungan yang selektif terutama lingkungan yang bersalinitas tinggi (hypersaline). Beberapa spesies mikroalga yang berhasil dikultur massal di outdoor dalam jangka panjang menggunakan saline-hypersaline media termasuk *Amphora coffeaeformis* MUR158 yang dilakukan oleh Mercz (1994), *Tetraselmis* spp(Fon-Sing and Borowitzka 2016), *Pleurochrysis carterae* (Moheimani and Borowitzka 2006, 2007), *Amphora* sp MUR 258 (Indrayani 2017). Spesies-spesies tersebut diatas merupakan spesies-spesies unggul dan sangat potensil untuk dikembangkan sebagai biodiesel feedstock karena produktivitas lipidnya tinggi serta dapat dikultur massal di outdoor dalam jangka waktu lama sehingga dapat diproduksi sepanjang tahun.

BAB 3. TUJUAN DAN MANFAAT PENELITIAN

Penelitian ini bertujuan untuk mendapatkan species lokal microalgae laut yang memiliki kandungan lipid yang tinggi, pertumbuhan yang cepat serta dapat dikultur secara massal di outdoor menggunakan media dengan salinitas tinggi (≥ 3% NaCl) untuk produksi biodiesel. Untuk mencapai tujuan tersebut maka akan dilakukan beberapa tahapan kegiatan yakni 1). Mengisolasi species lokal mikroalga laut dari berbagai habitat perairan asin termasuk estuaria dan pantai/ laut (selesai 2017), 2). Melakukan skrining/seleksi terhadap isolate microalgae yang memiliki pertumbuhan yang cepat, kandungan lemak yang tinggi serta kemampuannya untuk hidup pada salinitas yang tinggi (>3% NaCl) (on going 2018), 3). Melakukan ujicoba kultur massal di outdoor open pond system (raceway ponds) selama kurang lebih 6 bulan terhadap species-species yang unggul (2-3 species) (next 2019).

Target luaran (output) dari penelitian ini adalah 1). Akan diperoleh puluhan hingga ratusan isolate species lokal mikroalga laut yang selanjutnya akan disimpan dan dikembangkan untuk dipersiapkan sebagai cikal bakal pembentukan koleksi kultur mikroalga pada Fakultas Perikanan dan Ilmu Kelautan Universitas Haluoleo dan juga sebagai bahan/material penelitian dan pengembangan microalgae untuk aplikasi komersial". 2). Rekomendasi species/strain yang paling potensil sebagai biodiesel feedstock (2-3 species). 3). Akan dihasilkan beberapa publikasi baik pada jurnal nasional maupun jurnal international. 4). Hasil penelitian akan dipresentasikan pada konferensi Nasional dan International. 5). Akan memperkaya bahan ajar pada kuliah planktonologi, budidaya pakan alami, bioteknologi perairan dan bioprospecting kelautan.

BAB 4. METODE PENELITIAN

Tahapan Penelitian

Penelitian ini direncanakan akan berlangsung selama 3 tahun dengan tahapan sebagai berikut:

Tahun 1. Isolasi dan Identifikasi (selesai 2017)

Survey Lokasi Pengambilan Sampel air/mikroalga

Survey dilakukan di lokasi-lokasi yang dianggap sesuai dengan target capaian yakni isolasi mikroalga laut spesies lokal di perairan Kendari Sulawesi Tenggara termasuk di perairan pantai dan muara sungai. Lokasi-lokasi yang dimaksud adalah Muara Sungai Wanggu, Pantai Tanjung Tiram, Pantai Nambu, Pantai Batu Gong, Pantai Toronipa dan Pulau Bokori.

Persiapan alat dan bahan.

Melakukan pembelian dan pemesanan peralatan dan bahan-bahan dasar yang akan digunakan untuk keperluan isolasi seperti bahan untuk pembuatan media, wadah kultur serta menyiapkan ruang kultur untuk inkubasi/menumbuhkan mikroalga termasuk rak kultur, lampu untuk penerangan/sumber cahaya dilengkapi dengan alat timer (pengatur waktu lampu on dan off).

Pembuatan media kultur

Media kultur yang dipersiapkan adalah f/2 medium (Guillard and Ryther 1962). Agar medium dibuat dengan menambahkan 1% agar ke dalam liquid f/2 medium. Air laut yang digunakan untuk pembuatan media sebelumnya difilter melalui tank filter yang berisi kapas dan arang yang disusun secara berlapis dan berselang-seling. Salinitas media disesuaikan dengan salinitas lokasi pengambilan sampel.

Jenis medium kultur yang digunakan adalah f/2 medium. Medium kultur yang dibuat ada 2 jenis yakni agar f/2 medium dan liquid f/2 medium (medium cair). Prosedur pembuatan medium kultur adalah sebagai berikut :

1. Pembuatan stok solution f/2 medium.
   Untuk pembuatan stok solution f/2 medium digunakan air aquadest untuk melarutkan nutrient. Stok solution yang dibuat adalah nitrat NaNO3, phosphat NaH2PO4, Silika Na2SiO3, trace elements dan vitamin. Masing-masing stok solution dibuat terpisah dengan konsentrasi yang berbeda-beda yakni nitrat NaNO3 (75g/l), phosphat NaH2PO4 (5.65g/l), Silika NaSiO3 (30g/l) dan trace elements.
2. Pembuatan f/2 medium.
   Air laut yang diperoleh dari masing-masing lokasi digunakan untuk pembuatan bahan dasar f/2 medium. Air laut terlebih dulu disaring dengan kapas dan filter untuk mendapatkan air laut yang bersih dari kotoran-kotoran dan sebagian besar kontaminan baik zooplankton maupun mikroalga. Air laut yang telah difilter kemudian ditambahkan dengan nutrient dari stok solution f/2 medium termasuk nitrat (NaNO3), phosphat (NaH2PO4), Silika (NaSiO3) dan trace elements dengan dengan dosis untuk f/2 medium yakni masing 1 mL/1 Liter medium kultur. Untuk pembuatan medium agar, liquid medium ditambahkan dengan 1% agar (Merck). f/2 medium baik yang liquid maupun yang agar selanjutnya di autoclave pada suhu 121^{o}C. Setelah di autoclave, liquid medium didinginkan semalaman sebelum digunakan sementara untuk agar medium didiamkan sekitar 10 menit baru kemudian di tuang ke cawan petri dalam kondisi steril. Setelah medium agar dingin baru kemudian disimpan di lemari pendingin untuk digunakan sewaktu-waktu.

Pengambilan Sampel air untuk keperluan isolasi

Pengambilan sampel dilakukan di 5 lokasi yakni pantai Tanjung Tiram, pantai Nambo, pantai Batu Gong, pantai Toronipa dan pulau Bokori. Hal-hal yang dilakukan saat sampling pada masing-masing lokasi adalah :

1. Pengambilan sampel air dengan menggunakan plankton net. Tujuan dari pengambilan sampel plankton adalah untuk mendapatkan data tentang jenis-jenis dan

kelimpahan phytoplankton yang merupakan mikroalga planktonik (yang melayang-layang di dalam perairan).

2. Pengambilan sampel air tanpa menggunakan plankton net yang dimasukkan ke dalam botol sampel volume 1500 mL (sebanyak 3 botol untuk masing-masing lokasi). Sampel air ini digunakan untuk keperluan isolasi dengan metode enrichment/ pengkayaan dengan nutrient dan juga sebagai bahan pembuatan media kultur (f/2 medium).
3. Pengukuran parameter kualitas air termasuk suhu, salinitas, pH, kecerahan, kedalaman (in situ) serta pengambilan sampel air untuk keperluan analisa nitrat, phosphat dan ammonia (ek situ).

Isolasi dan inkubasi isolat

Isolasi dilakukan menggunakan metode isolasi langsung, pengenceran dan penggoresan pada media agar plate dengan mengacu pada Andersen and Kawachi (2005). Sample diinkubasi pada intensitas cahaya rendah (20-30 µmol photon.m^{-2}.s^{-1}) dengan siklus gelap dan terang (12 jam: 12 jam) pada suhu kamar 27-30ºC.

Biakan murni dan skale-up

Isolat yang didapat selanjutnya akan ditransfer ke 24-microtitter well plate yang berisi 2 mL medium dan selanjutnya diinkubasi pada intensitas 50-70 µmol photon.m^{-2}.s^{-1}, siklus 12 jam gelap:12 jam terang pada suhu kamar. Liquid kultur selanjutnya di scale-up dari 2 mL – 10 mL -50 mL – 100 mL – 500 mL dan 1 L sehingga didapatkan cukup inoculum untuk tahapan selanjutnya (Skrining).

Identifikasi. Identifikasi dilakukan terhadap spesies-spesies mikroalga yang berhasil dikultur monospesies (unialgal culture) dan di skale-up dengan mikroskop berdasarkan jenis pigmen, ciri-ciri morphologi termasuk bentuk sel (filament, bulat atau rod-like), ada atau tidaknya flagel, phyrenoid dan feature lainnya dengan mengacu pada buku identifikasi phytoplankton/mikroalga.

Tahun 2. Skrining (on going 2018)

Adapun tahapan-tahapan kegiatan penelitian pada tahun ke-2 adalah sebagai berikut :

1. Skrining awal species/strains yang dapat dikultur pada hypersaline media

- Biakan murni dari isolat yang telah dihasilkan akan diskale up ke 300 mL untuk mendapatkan cukup inokulum untuk eksperimen skrining awal
- Strains yang mudah dikultur dan di skale up selanjutnya di kultur dalam Erlenmeyer 50 mL berisi 30 mL f/2 medium pada salinitas yang berbeda yakni 3,4 dan 5% NaCl (w/v), intensitas 70 µmol photon.m-1.s-1, siklus 12 jam gelap :12 jam terang pada suhu kamar. Species yang tumbuh baik pada salinitas 5% NaCl selanjutnya akan dipilih untuk eksperimen selanjutnya. Hal ini dilakukan sebagai skrining awal spesies yang dapat dikultur pada hypersaline media.

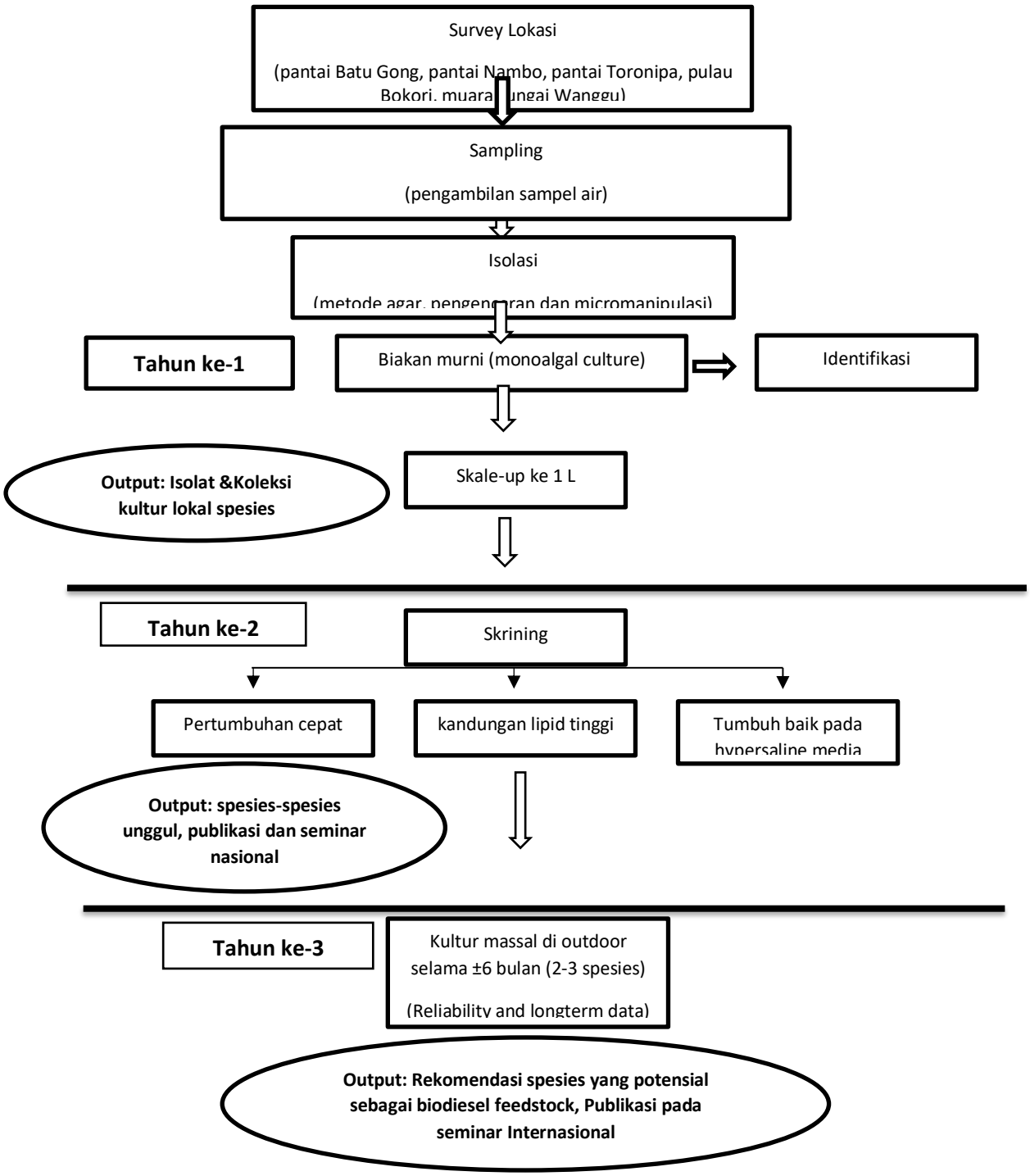
2. Eksperiment pengaruh salinitas yang berbeda terhadap pertumbuhan dan kandungan lipid mikroalga

- Dari hasil skrining awal, spesies yang terpilih selanjutnya akan dikultur dalam erlemenyer 300 mL berisi 150 mL f/2 medium pada intensitas 70 µmol photon.m-1.s-1, siklus 12 jam gelap :12 jam terang pada suhu kamar pada salinitas 3, 4, 5 dan 6% NaCl (in triplicates).
- Sampling untuk mengukur pertumbuhan (penghitungan jumlah sel dan berat kering biomass) dilakukan setiap 2 hari sedangkan untuk analisa kandungan lipid dilakukan pada hari ke 2-4 (ekponensial phase) dan hari ke 12-15 (stationary phase)
- Karakterisasi komposisi fatty acids (asam lemak) dari isolat yang tumbuh dengan baik pada salinitas hingga 6%
- Species yang paling potensil yang memiliki pertumbuhan yang bagus/cepat pada salinitas tinggi serta kandungan lipid yang tinggi akan dipilih untuk di kultur secara massal di outdoor.

Tahun 3. Kultur massal di outdoor (2019)

.Kultur outdoor menggunakan raceway pond (fibreglass) berukuran 1 m^2 (2×0.5×0.4 m, L×W×D) dan dilengkapi dengan paddlewheels. Species unggul selanjutnya dikultur menggunakan air laut yang telah difilter dan sudah diperkaya dengan nutrient f/2 medium sedangkan salinitasnya diatur sesuai dengan salinitas optimum species yang dikultur. Inokulum untuk kultur massal dipersiapkan di indoor menggunakan beberapa buah carboys volume 20 L.

Adapun bagan alir penelitian adalah :



Metode Analysis

Penghitungan jumlah sel

Pertumbuhan kultur dimonitor dengan menghitung jumlah sel menggunakan Neubauer haemocytometer (Moheimani et al. 2013).

Menentukan laju pertumbuhan specific (Specific growth rate)

Laju pertumbuhan specific (specific growth rate (μ)) dihitung menggunakan persamaan berikut:

$$\boldsymbol{\mu = \frac{Ln\,(N_2\,/N_1\,)}{t_2\,-\,t_1}}$$

dimana N_1 and N_2 adalah kepadatan sel pada waktu ke 1 (t_1) and 2 (t_2).

Menentukan berat kering biomass (Dry weight (DW) dan berat kering organic (ash free dry weight (AFDW))

Penetuan DW dan AFDW mengacu pada standar metode untuk mengukur pertumbuhan microalgae (Moheimani et al. 2013):

$$\boldsymbol{Dry\ weight\ (g.L^{-1}) = (weight\ of\ filters\ plus\ algae) - (weight\ of\ filters)}$$

Filter kemudian dipindahkan ke oven furnace pada suhu 450^{o}C selama 5 jam. Berat kering 14rganic (Ash-free dry weight) dihitung menggunakan formula berikut:

$$\boldsymbol{Ash - freedryweight(g.L^{-1}) = Dryweight - weightafterashing}$$

Lipid Extraction

Ekstraksi lipid menggunakan metode Bligh and Dyer (1959)yang dimodifikasi oleh Kates and Volcani (1966) dan diadaptasi olehMercz (1994).

Analisa Data

Eksperimen pengaruh salinitas dilakukan dalam triplicate. One-way analysis of variance (ANOVA) digunakan untuk melihat perbedaan dari masing-masing perlakuan. Jika uji normalitas dan equal variance test gagal maka akan digunakan the Holm-Sidak pairwise comparison method based on ranks. Semua analisa statistik akan menggunakan Sigma-Plot 13 package.

BAB 5. HASIL YANG DICAPAI DAN LUARAN

5.1. Hasil

5.1.1. Skrining awal species/strains yang dapat dikultur pada hypersaline media

Out of eight isolates tested for their ability to grow at high salinity, three isolates namely isolate IND-UHO-029, IND-UHO-003 and IND-UHO-002 showed very good growth over wide ranges of salinity tested (3-5% NaCl) and showed no lag phase following inoculation indicating that they can adapt well at high salinity (Figure 1).

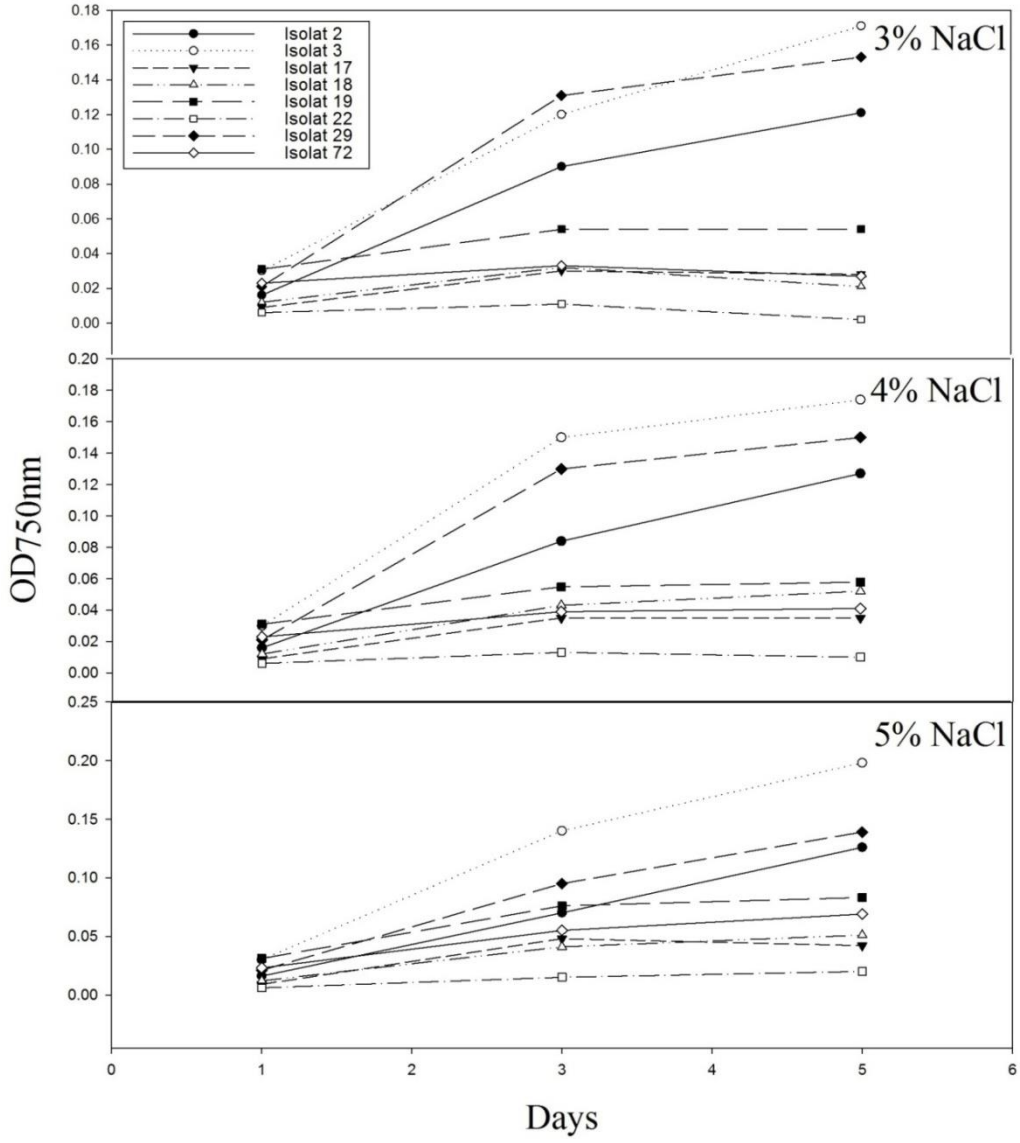


Figure 1. Growth curves of the newly isolated microalgae from Kendari Waters at different salinity

The specific growth rates of eight isolates under different salinity are shown in the Figure 2. The isolate IND-UHO-029 had the higest specific growth rate at salinity 3 and 4% NaCl followed by isolates IND-UHO-002 and IND-UHO-003. However, at 5%NaCl, the three isolate had about the same specific growth rate of about 0.74 - 0.77d^{-1}. Isolates IND-

UHO-017 and IND-UHO-018 showed similar pattern in which the specific growth rate increased and reached maksimum at 4% salinity of about 0.68 and 0.64 d^{-1}, respectively before decreasing at 5% to about 0.64 and 0.61d^{-1}, respectively. It is interestingly to note that the specific growth rates of isolate IND-UHO 019, IND-UHO 022 and IND-UHO 72 increased as the salinity increase.

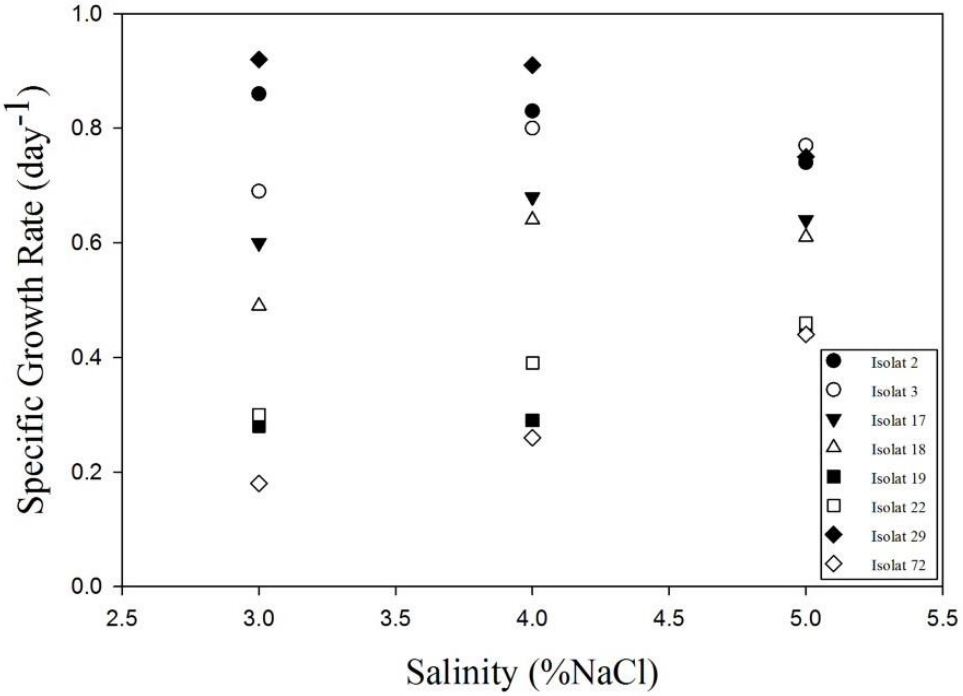


Figure 2. Specific Growth Rates (day^{-1}) of the newly isolates microalgae from Kendari Waters under different salinity

5.1.2. Eksperiment pengaruh salinitas yang berbeda terhadap pertumbuhan dan kandungan lipid mikroalga

5.1.2.1. Strain *Nannochloropsis* sp IND-UHO 003

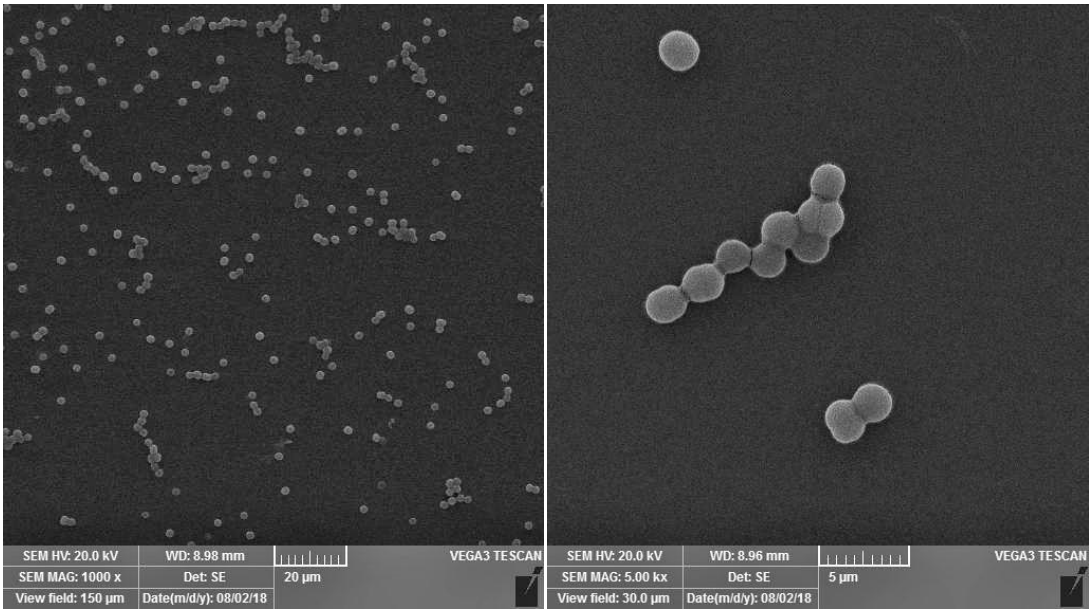


Figure 3. Scanning Electron Microscopy Images of the *Nannochloropsis* sp IND-UHO-003 at 1000x Maginification (left) and 10000x Magnification (right)

Growth of the *Nannochloropsis* IND-UHO 003

The growth of the *Nannochloropsis* for three weeks of culturing at increasing salinity under semi-continuous regime is shown in **Figure 4**. The alga showed very good growth following initial inoculation at 3% salinity and after the salinity of the cultures increased gradually up to 7% NaCl (w/v).

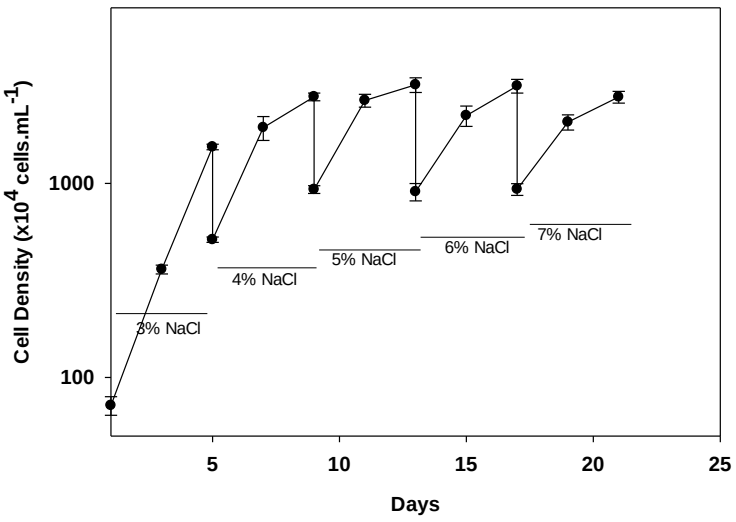


Figure 4. Growth of the *Nannochloropsis* sp IND-UHO 003 at increasing salinity under semi-continuous mode

The specific growth rates of the *Nannochloropsis* decreased at increasing salinity. The highest specific growth rate (0.73±0.029 d^{-1}) was obtained at 3% salinity and the lowest (0.40±0.02 d^{-1}) was at 7% salinity (One Way ANOVA, $F_{(4,10)}$ =62.025, p<0.001) (**Figure 5**).

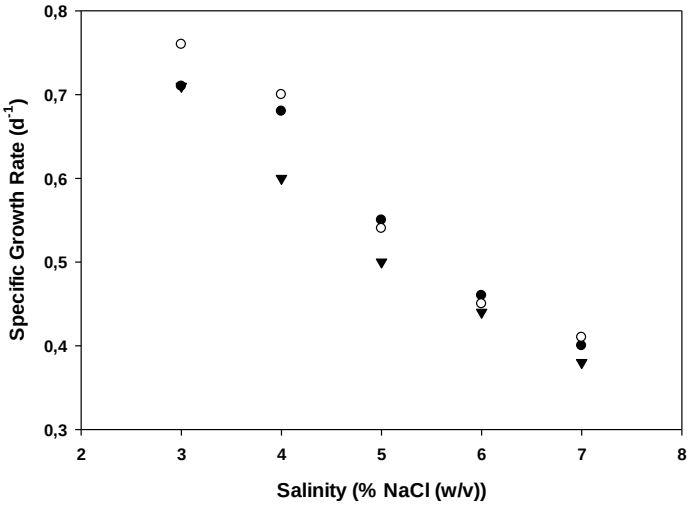


Figure 5. Specific growth rate (d^{-1}) of the *Nannochloropsis* sp. IND-UHO 003 at different salinity. Symbols represent the three replicate cultures

Biomass yield and productivity of the *Nannochloropsis* IND-UHO 003

Biomass yield of the *Nannohloropsis* was affected by the salinity tested. The highest biomass yield (1.10±0.1 $g.L^{-1}$) achieved at 4% salinity and the lowest (0.55±0.1 $g.L^{-1}$) obtained at 3% salinity (One Way ANOVA, $F_{(4,10)}$=21.243,p<0.001). Simiarly, the highest biomass productivity achieved at 4% salinity (0.73±0.12 $g.L^{-1}.d^{-1}$) and the lowest was at 3% salinity (0.39±0.07 $g.L^{-1}.d^{-1}$) (One Way ANOVA, $F_{(4,10)}$=12.690,p<0.001). It is important to note that both biomass yield and biomass productivity increased more than two fold when the salinity was increased from 3% to 4% NaCl (**Figure 6**).

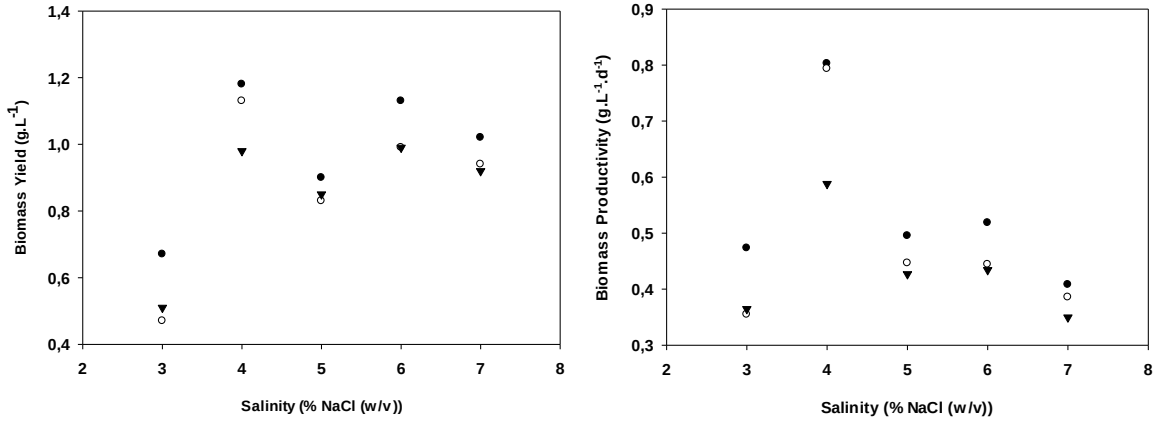


Figure 6. Biomass yield ($g.L^{-1}$) and Biomass productivity ($g.L^{-1}.d^{-1}$) of *Nannochloropsis* sp. IND-UHO 003 growing under different salinity. Symbols show the three replicate cultures.

Lipid of *Nannochloropsis* IND-UHO 003

The lipid yield of the Nannochloropsis ranged from 0.087±0.011 $g.L^{-1}$ to 0.167±0.011 $g.L^{-1}$. The highest lipid achieved at 5% salinity and the lowest occured at 4% salinity (One Way ANOVA, $F_{(4,10)}$=13.670, p<0.001) (**Figure 7**). The alga achieved its highest lipid content when grown at 3% salinity (25.05±1.9% AFDW) and the lowest lipid content achieved when the alga was grown at 4% salinity (8.11±0.75% AFDW) (One Way ANOVA, $F_{(4,10)}$=55.055, p<0.001) (Figure 3). The lipid productivity of the alga reached maksimum at 3% salinity (1.10± 0.011 $g.L^{-1}.d^{-1}$) and the lowest lipid productivity obtained at the highest salinity 7% NaCl (0.036±0.003 $g.L^{-1}.d^{-1}$) (One Way ANOVA, $F_{(4,10)}$=32.177, p<0.001).

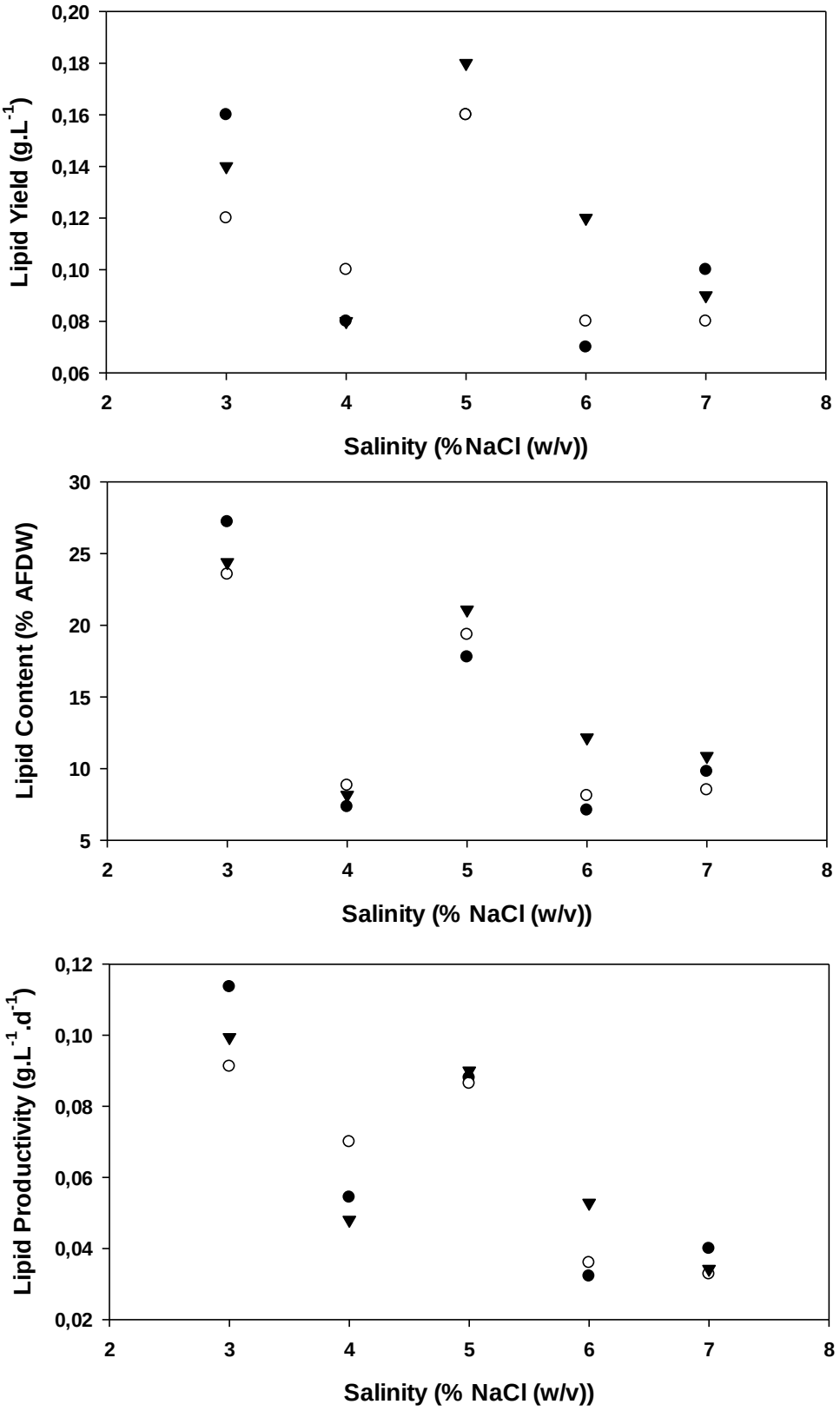


Figure 7. Lipid yield (g.L^{-1}), lipid content (% AFDW) and Lipid productivity (g.L^{-1}.d^{-1}) of *Nannochloropsis* sp. IND-UHO 003 at different salinity. Symbols show the three replicate cultures.

5.1.2.2. Strain *Skeletonema* sp IND-UHO 029

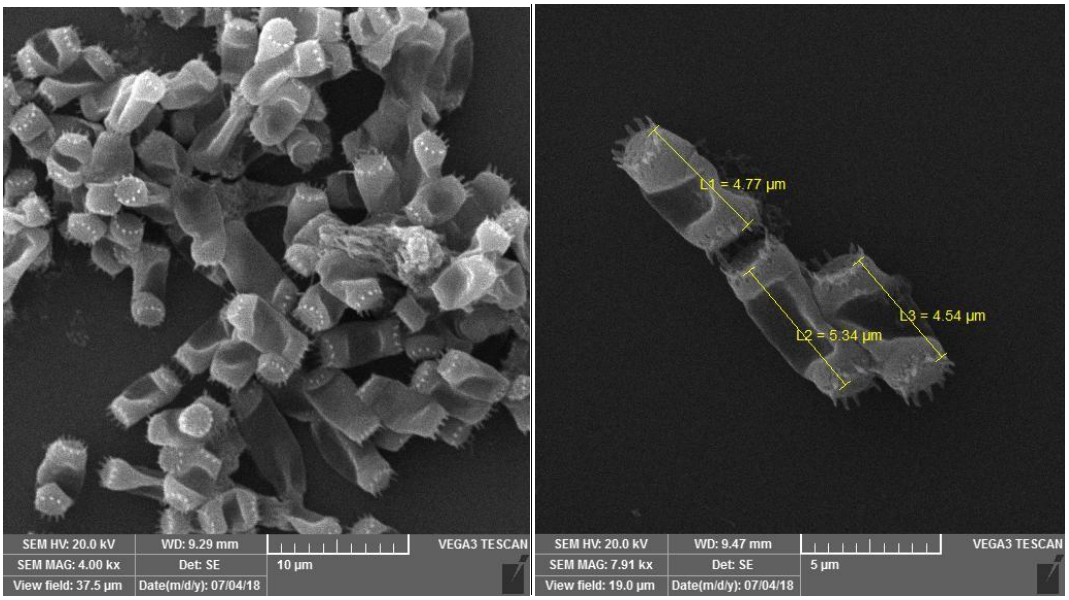


Figure 8. Scanning Electron Microscopy Images of the *Skeletonema* sp IND-UHO 029 at 4000x Maginification (left) and 8000x Magnification (right)

Table 1. SGR, Biomass Yield and Biomass Productivity of *Skeletonema* sp IND-UHO 029 at different salinity

Salinitas (%)	SGR (d-1)	Biomass (g AFDW.L-1)	Biomass Productivity (g AFDW.L-1.d-1)
3	0,483±0,015	0,850±0,172	0,319±0,094
4	0,520±0,02	0,950±0,096	0,494±0,057
5	0,450±0,017	0,876±0,019	0,407±0,023
6	0,363±0,040	0,951±0,061	0,408±0,060

Table 2. Lipid Yield, Lipid Content and Lipid Productivity of *Skeletonema* sp IND-UHO 029 at different salinity

Salinitas (%)	Lipid Yield (g.L-1)	Lipid Content (%AFDW)	Lipid Productivity (g.L-1.d-1)
3	0,173±0,030	28,72±5,24	0,084±0,016
4	0,213±0,080	24,74±4,64	0,112±0,046
5	0,193±0,042	22,07±5,34	0,087±0,015
6	0,180±0,040	18,29±3,65	0,065±0,011

Table 3. SGR, Biomass yield and biomass productivity of Skeletonema sp IND-UHO 029 under different light intensity and N:P ratios

Treatment	SGR (d-1)	Biomass (g AFDW.L-1)	Biomass Productivity (g AFDW.L-1.d-1)
Low Light	0,25±0,04	0,07±0,014	0,018±0,006
Medium Light	0,44±0,01	0,425±0,021	0,185±0,006
High Light	0,49±0,08	0,39±0,00001	0,191±0,033
1N:1P	0,435±0,007	0,425±0,021	0,185±0,006
2N:1P	0,45±0,014	0,485±0,021	0,218±0,016
3N:1P	0,46±0,028	0,38±0,00001	0,175±0,011
1N:2P	0,335±0,035	0,325±0,064	0,11±0,033

Table 4. Lipid yield, lipid content and lipid productivity of Skeletonem sp IND-UHO 029 under different light inetnsity and N:P ratios

Treatment	Lipid Yield (g.L-1)	Lipid Content (%AFDW)	Lipid Productivity (g.L-1.d-1)
Low Light	0,02±0,00001	29,17±5,89	0,005±0,0008
Medium Light	0,080±0,00001	18,85±0,94	0,0348±0,0005
High Light	0,060±0,00001	15,38±2,92	0,0294±0,005
1N:1P	0,080±0,00001	18,85±0,94	0,0348±0,0005
2N:1P	0,120±0,028	24,89±6,92	0,0538±0,011
3N:1P	0,100±0,028	26,32±7,44	0,0464±0,0158
1N:2P	0,040±0,00001	12,55±2,46	0,0134±0,0014

5.2. Luaran

Dari hasil penelitian yang telah dicapai sejauh ini telah dihasilkan beberapa luaran sebagai berikut :

1. Abstrak berjudul “Isolation and Screening of Marine Microalgae from Kendari Waters, Southeast Sulawesi, Indonesia Suitable for Outdoor Mass Cultivation in Hypersaline Media” telah dipresentasikan pada “Symposium Nasional Makro dan Mikroalga” yang diselenggarakan oleh Ikatan Fikologi Indonesia pada tanggal 8 Agustus 2018 di Hotel Arthama, Makassar (sertifikat terlampir)
2. Article berjudul “Isolation and Screening of Marine Microalgae from Kendari Waters, Southeast Sulawesi, Indonesia Suitable for Outdoor Mass Cultivation in Hypersaline Media” telah disubmit pada Jurnal Internasional AACL Bioflux (Artikel terlampir)
3. Draft artikel berjudul “Effects of salinity on the growth and lipid production of A Newly Isolated marine microalga *Nannochloropsis* sp IND-UHO 003 akan disubmit pada jurnal internasional (Artikel terlampir)
4. Abstrak berjudul “Biomass and Lipid Productivities of A Newly Isolated Diatom (*Skeletonema* sp IND-UHO-029) at Increasing Salinities” akan dipresentasikan pada seminar internasional”The 2nd ICTROPS” yang akan diselenggarakan oleh Universitas Mulawarman di Balipkpapan pada tanggal 18-19 September 2018.

BAB 6. RENCANA TAHAPAN BERIKUTNYA

Rencana tahapan berikutnya adalah sebagai berikut :

1. Melakukan karaketrisasi komposisi asam lemak (fatty acids) dari ketiga isolat (IND-UHO 002, 003 dan 029)
2. Identifikasi Molekular ke 3 jenis isolat (IND-UHO 002, 003 dan 029)
3. Seminar Internasional (the 2nd ICTROPS, Balikpapan 18-19 September 2018)
4. Submit artikel kedua ke jurnal internasional
5. Pembuatan laporan akhir

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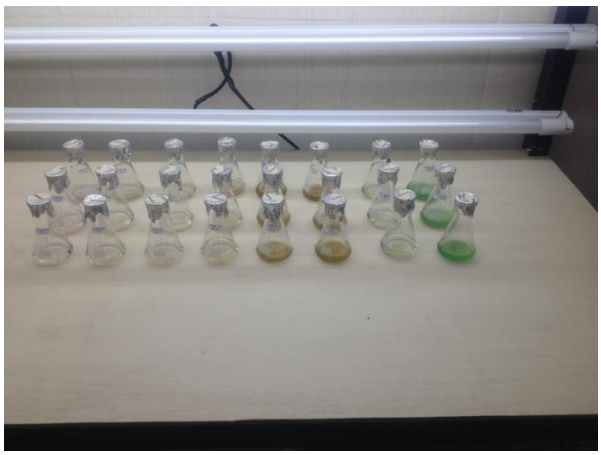
LAMPIRAN

1. Penyaringan Air Laut untuk bahan dasar media kultur

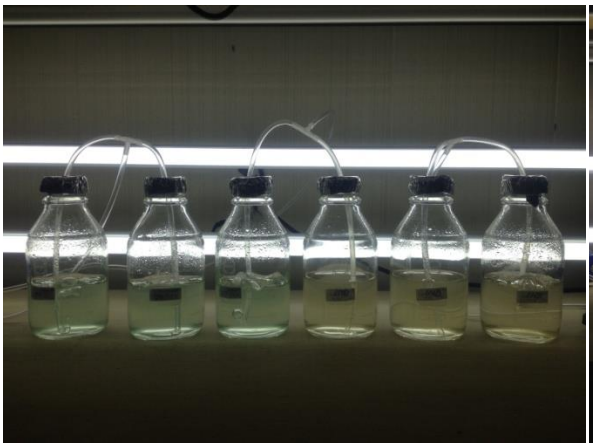
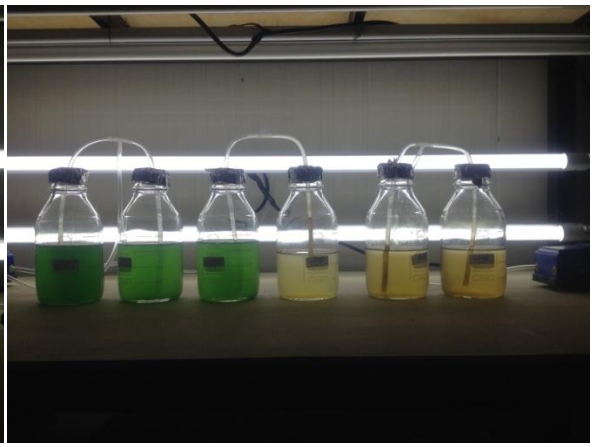

2. Persiapan inokulum kultur untuk skrining awal


3. Skrining awal 8 isolates (IND-UHO 002, 003, 016, 017, 019, 021, 029, 072) di kultur pada salinitas 3,4 dan 5% NaCl dengan tiga ulangan

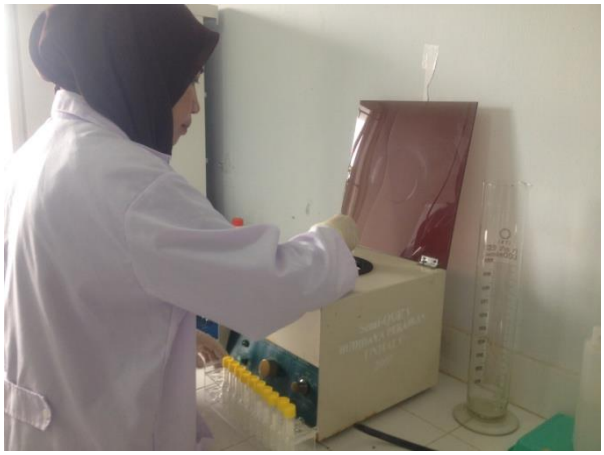
4. Pengamatan kondisi kultur skrining awal dan penghitungan kelimpahan sel


5. Kultur Isolat IND-UHO 002 dan IND UHO 003 pada salinitas yang berbeda (3,4,5 dan 6% NaCl) dengan 3 ulangan. Kultur hari ke 1 (kiri) hari ke 5 (kanan)

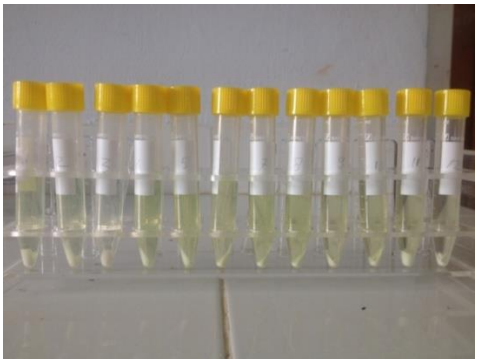



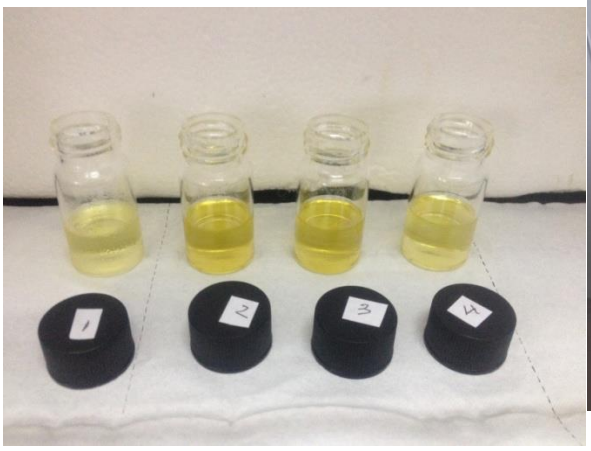
6. Sampling penentuan Dry Weight. Penimbngan berat filter (kiri), filter sampel kultur (kanan), oven 75°C selama 4 jam (bawah)


7. Ekstraksi Lipid. Pemecahan dinding cell dengan menggerus menggunakan glass rod (atas), Centrifugasi sampel (kiri bawah), vortexing sampel (kanan bawah)




8. Ekstraksi lipid (lanjutan). Lipid sampel dalam larutan campuran kloroform, metanol dan air (kiri atas), terjadi pemisahan antara larutan metanol dan air pada lapisan atas dan lipid dan kloroform pada lapisan bawah (kanan atas). Pemisahan antara lapisan atas dan bawah (kanan tengah), lipid terlarut dalam kloroform setelah pemisahan (kiri tengah). Evaporasi solvet hingga kering(kiri bawah), penimbangan berat lipid (kanan bawah)



9. Paper 1 (Submitted to Jurnal Internasional, AACL Bioflux)

Isolation and Screening of Marine Microalgae from Kendari Waters, Southeast Sulawesi, Indonesia Suitable for Outdoor Mass Cultivation in Hypersaline Media

Indrayani[1*], Haslianti[1] and Asriyana[1]

[1]Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Halu Oleo University, Kampus Bumi Tridharma Anduonohu, Kendari 93232, Southeast Sulawesi, Indonesia

*Corresponding Author: email: indrayani_tajudin@yahoo.com.au, phone: 082188629424

Abstract

Screening of local species microalgae for any commercial application has a competitive advantage as they are well adapted to the local climatic environment. Microalgae capable to grow in hypersaline media are particularly interesting as they are more sustainable to grow and less prone to contaminations. The aim of this study was to isolate and screen local species microalgae from Kendari Waters Southeast Sulawesi Indonesia suitable for mass cultivation in outdoor open pond systems in hypersaline media. The water samples for isolation of microalgae were collected from several coastal areas in Kendari, Southeast Sulawesi including Tanjung Tiram Beach, Nambo Beach, Batu Gong Beach, Toronipa Beach and Bokori Island. The isolation of microalgae was done using agar plating technique (1% agar in f/2 medium). The water samples were plated on agar medium and incubated under low light, 12:12 hours light and dark cycle and at ambient room temperature. There are hundreds of isolates generated after repeated streaking on agar medium. The isolates are dominated by diatom and cyanobacteria. Three isolates (IND-UHO-002, IND-UHO-003 and IND-UHO-029) showed fast growth at high salinity (5% NaCl) and hence are promising strains for mass cultivation in outdoor open pond system in hypersaline media.

Keywords: Hypersaline media, isolation, microalgae, Southeast Sulawesi

Introduction

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can be found in all ecosystems both aquatic and terrestrial (Richmond 2004; Mata et al. 2010). They are an extremely heterogeneous group of microorganisms which are potentially rich source of important chemicals with potential application in the feed, food, nutritional, cosmetics, pharmaceuticals and even in fuel industries (Olaizola 2003). Although, there are over thousands or even millions of microalgae species exist in nature, only a few of them have been successfully produced commercially for the production of high value

products (i.e. *Dunaliella salina* for β-carotene, *Haematococcus pluvialis* for astaxanthin and *Crypthecodinium cohnii* for EPA production). Hence, bioprospecting of microalgae species with commercial potential is thus an important and challenging task.

Species or strain selection is the first and critical step in bioprospecting of microalgae for any commercial application (Barclay and Apt 2013; Borowitzka 2013a). Screening of microalgae species involves a series of steps including sample collection, isolation, purification, identification, maintenance and characterization of potential products (Gong and Jiang 2011). There are two possible sources of selecting/screening microalgae; from microalgae culture collections and from natural environments. Species selection through microalgae culture collections can be accessed easily although the number of microalgae species kept in the culture collection is only a small fraction of microalgae species that exist in nature (Borowitzka 2013a). On the other hand, untapped resources of microalgae species can be isolated from natural environments. Isolating and selecting of local microalgae species/strains has a competitive advantage especially for microalgae species intending to be mass produced in outdoor as they are well adapted to the local climatic environment (Larkum et al. 2012).

Marine microalgae that can grow in saline-hypersaline media are particularly interesting because they are more sustainable and economical to grow compared to freshwater microalgae. Marine microalgae use seawater that can be accessed easily and freely by just pumping the seawater from coastal areas whereas freshwater microalgae require limited and precious freshwater . Marine microalgae can be grown in non-arable lands and therefore will not compete for agriculturals (Borowitzka and Moheimani, 2013b). Moreover, hypersaline microalgae are less prone to contaminations by other microorganisms (i.e microalgae, protozoas and bacteria) due high salt concentration of culture medium where not many organisms can tolerate. Therefore, the aim of this study was to isolate local species microalgae from Kendari Waters Southeast Sulawesi Indonesia and to screen them for potential mass cultivation in outdoor open pond systems in saline-hypersaline media for any commercial applications. This study is the first to isolate local species microalgae from several coastal areas in Kendari and to initiate the establisment of the microalgae culture collection in Kendari Southeast Sulawesi, Indonesia.

Materials and Methods

Sampling Sites

The sampling sites for this study were conducted at several coastal areas in Kendari, Southeast Sulawesi, Indonesia including Tiram Cape (4°2’24”BT-122°40’1”LS), Nambo Beach (4°0’5”BT-122°36’58”LS), Batu Gong Beach (3°52’43”BT-122°30’45”LS), Toronipa Beach (3°54’0”BT-122°39’36”LS) and Bokori Island (3°55’34”BT-122°40’0”LS).

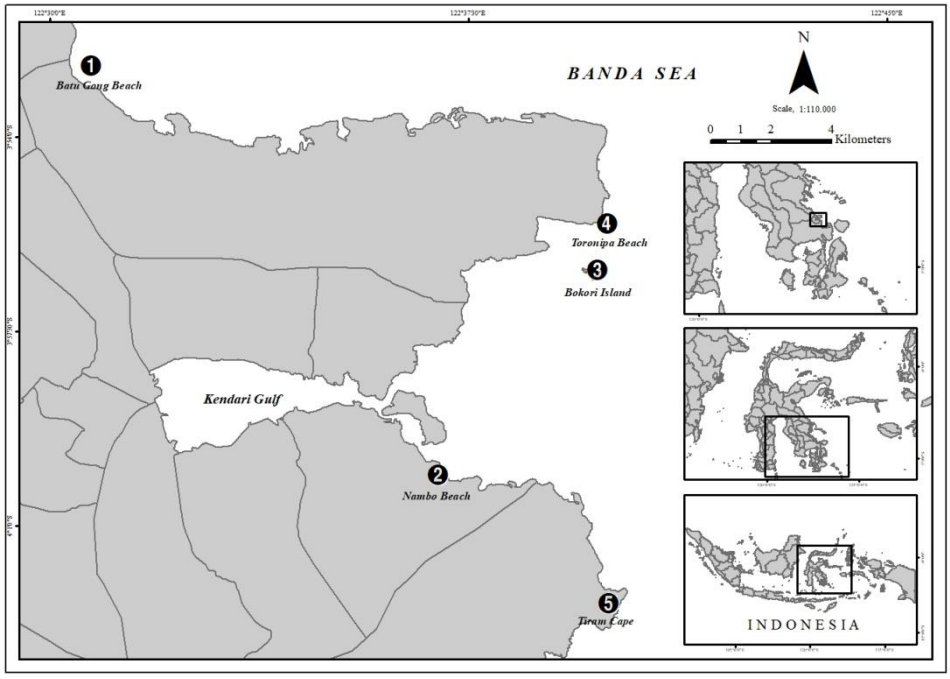


Figure 1. Sampling Sites

Sample collection

Water samples were collected manually using plastic water bottles volume 1.5 L and also using a plankton net mesh size 25 µm. For the plankton net, about 50 litres of seawater at each location were filtered through the plankton net and collected in a 50 mL tube. In the lab, the water samples from each location were transferred into small glass bottles (100 mL) and enriched with nutrients (nitrat, phosphate, silicate and trace elements) based on f/2 medium stock solution (Guillard and Ryther 1962) and then incubated in a culture shelve with illumination of a 20 watt of white fluorescent tubes under 12:12 hour light and dark cycles and at ambient room temperature whereas the sample from plankton net was prepared for isolation straight away.

Isolation and Identification of Microalgae

Isolation of the microalgae was carried out using agar plating technique (Andersen and Kawachi 2005). The medium used was f/2 medium (Guillard and Ryther 1962). Agar medium was prepared by adding 1% of agar into the liquid medium prior to autoclaving.

Sample from plankton net (0.1 mL) was spotted on the middle of the agar plates and spread evenly on the surface using a glass spreader. The plates were then sealed with parafilm to avoid drying out and incubated in the culture shelve under dim light (a 20 watt of white fluorescent tube light) with 12 h light and 12 h dark cycle and at room temperature. The same protocols were applied for the enrichment samples.

Colonies emerged on the agar plates were picked up and re-streaked onto fresh agar medium using standard microbiology agar streaking technique. Pure unialgal colonies were obtained after repeated streaking on fresh agar media and confirmed by microscopic observation. The pure colonies were then transferred into liquid medium by inoculating a single colony into each well of the 24 and 96-wells microtiter plate containing 1 mL and 200 µL of f/2 medium, respectively.

Identification of the microlagal isolates was based on the morphological characteristics using a microscope and phytoplankton identification books (Newell dan Newell 1977; Yamaji 1976). The isolates were identified up to genus level.

Screening of Microalgae suitable for mass cultivation in hypersaline media in outdoors

For screening, new isolates were grown in microtiter well plates and then gradually scale-up from 0.2 mL – 2 mL – 10 mL, 50 mL and 100 mL at ambient room temperature (28-34°C), 12 h:12h light and dark cycles and under light intensity of 2x20 watt of white florescent tube light (about 70-80 µmol.photon.m^{-2}.s^{-2}). The cultures were then observed for their ability to grow in liquid medium. The cultures that grew well in liquid medium were then selected for further screening test. About 30 mL aliquots of the cultures were then transferred to flasks containing 50 mL of f/2 medium at three different salinities (3,4 and 5% NaCl) and cultures for one week.

The growth of the cultures was monitored every two days by measuring the optical density of the cultures at OD750 nm using UV-Spectrometer. The specific growth rate (µ) was then calculated using the following equation (Moheimani et al. 2013) :

$$\boldsymbol{\mu = Ln\ (N_2\ /N_1)/t_2 - t_1}$$

Where N_1 and N_2 are the OD reading at time 1 (t_1) and 2 (t_2) within the exponential phase.

Results

Isolation of Microalgae

Hundreds of microalgal isolates (pure colonies) have been succesfully established after repeated streaking on agar f/2 medium (Figure 1a) and after growing on 24 and 96 micrtiter well-plates (Figure 1b). Out of hundreds of isolates on agar, about 77 isolates successfully grown in liquid medium (Figure 1c).

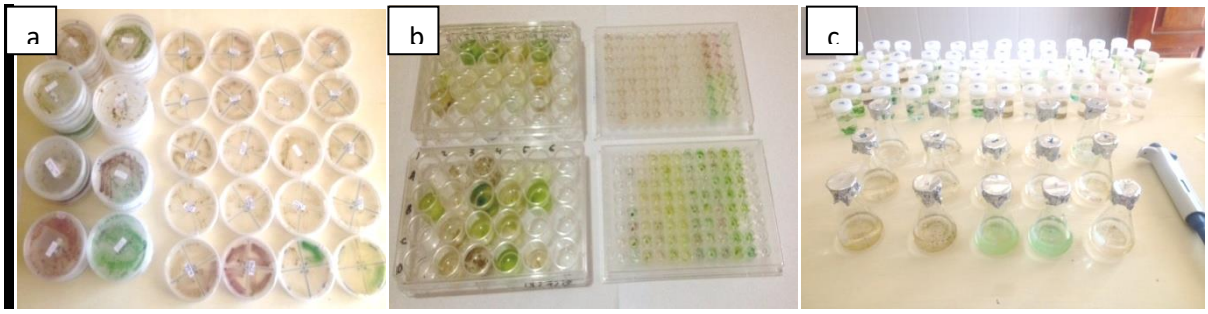


Figure 2. Isolates grown on agar plate (a), 24 and 96 microtiter well-plates (b) and small containers and flasks (c)

Identification of the microalgal species was only performed on the species that can grow in liquid medium. The isolates grown in liquid medium are dominated by diatoms/ Bacillariophyceae including *Diatoma* sp, *Navicula* sp, *Melosira* sp, *Synura* sp, *Surirella* sp), filamentous cyanobacteria (*Oscillatoria* sp and *Anabaena* sp) and green algae/Chlorophyte including *Chlorella* sp and *Chlamydomonas* sp (Table 1). Most of the diatom species isolated are from Bokori Island and Tanjung Tiram Beach whereas the filamentous cyanobacteria and the green algae are from Batu Gong, Toronia Beach and Nambo Beach.

Table 1. Identification of newly isolated local species microalgae in Coastal areas in Kendari Southeast Sulawesi Indonesia

Isolate Code	*Scientific Name*	Place of Collection
IND-UHO 001	*Diatoma sp*	Bokori Island
IND-UHO 002	*Diatoma sp*	Bokori Island
IND-UHO 003	*Chlorella sp*	Bokori Island
IND-UHO 004	*Diatoma sp*	Bokori Island
IND-UHO 005	*Navicula sp*	Tanjung Tiram Beach
IND-UHO 006	*Navicula sp*	Tanjung Tiram Beach
IND-UHO 007	*Navicula sp*	Tanjung Tiram Beach
IND-UHO 008	*Diatoma sp*	Tanjung Tiram Beach
IND-UHO 009	*Navicula sp*	Tanjung Tiram Beach
IND-UHO 010	*Navicula sp*	Tanjung Tiram Beach
IND-UHO 011	*Diatoma sp*	Bokori Island
IND-UHO 012	*Synura sp*	Bokori Island
IND-UHO 013	*Synura sp*	Bokori Island
IND-UHO 014	*Synura sp*	Bokori Island
IND-UHO 015	*Navicula sp*	Bokori Island
IND-UHO 016	*Navicula sp*	Bokori Island
IND-UHO 017	*Navicula sp*	Bokori Island
IND-UHO 018	*Nitzschia sp*	Bokori Island
IND-UHO 019	*Aphanocapsa sp*	Bokori Island
IND-UHO 020	*Tabellaria*	Bokori Island
IND-UHO 021	*Aphanocapsa sp*	Bokori Island
IND-UHO-022	*Porphyridium sp*	Bokori Island
IND-UHO-023	*Tetraselmis sp*	Bokori Island
IND-UHO 025	*Surirella sp*	Bokori Island
IND-UHO 028	*Diatoma sp*	Bokori Island

IND-UHO 029	*Melosira sp*	Nambo Beach
IND-UHO 030	*Melosira sp*	Nambo Beach
IND-UHO 031	*Oscillatoria sp*	Nambo Beach
IND-UHO 033	*Chlorella sp*	Bokori Island
IND-UHO 034	*Oscillatoria sp*	Batu Gong Beach
IND-UHO 035	*Oscillatoria sp*	Bato Gong Beach
IND-UHO 036	*Oscillatoria sp*	Toronipa Beach
IND-UHO 037	*Oscillatoria sp*	Toronipa Beach
IND-UHO 038	*Oscillatoria sp*	Nambo Beach
IND-UHO 039	*Dinobryon sp*	Nambo Beach
IND-UHO 040	*Melosira sp*	Nambo Beach
IND-UHO 041	*Oscillatoria sp*	Nambo Beach
IND-UHO 042	*Oscillatoria sp*	Nambo Beach
IND-UHO 043	*Oscillatoria sp*	Nambo Beach
IND-UHO 044	*Oscillatoria sp*	Nambo Beach
IND-UHO 045	*Oscillatoria sp*	Nambo Beach
IND-UHO 046	*Oscillatoria sp*	Nambo Beach
IND-UHO 051	*Oscillatoria sp*	Toronipa Beach
IND-UHO 052	*Oscillatoria sp*	Toronipa Beach
IND-UHO 053	*Oscillatoria sp*	Toronipa Beach
IND-UHO 054	*Oscillatoria sp*	Toronipa beach
IND-UHO 055	*Oscillatoria sp*	Toronipa Beach
IND-UHO 056	*Oscillatoria sp*	Toronipa Beach
IND-UHO 057	*Oscillatoria sp*	Batu Gong Beach
IND-UHO 058	*Navicula sp*	Batu Gong Beach
IND-UHO 059	*Anabaena sp*	Batu Gong Beach
IND-UHO 060	*Oscillatoria sp*	Batu Gong Beach
IND-UHO 061	*Oscillatoria sp*	Batu Gong Beach
IND-UHO 062	*Oscillatoria sp*	Batu Gong Beach
IND-UHO 063	*Navicula sp*	Batu Gong Beach
IND-UHO 064	*Melosira sp*	Tanjung Tiram Beach
IND-UHO 065	*Oscillatoria sp*	Tanjung Tiram Beach
IND-UHO 066	*Oscillatoria sp*	Tanjung Tiram Beach
IND-UHO 067	*Diatoma sp*	Nambo Beach
IND-UHO 068	*Navicula sp*	Nambo Beach
IND-UHO 069	*Coscinodiscus sp*	Toronipa Beach
IND-UHO 070	*Oscillatoria sp*	Batu Gong Beach
IND-UHO 072	*Chlamydomonas sp*	Tanjung Tiram Beach
IND-UHO 073	*Diatoma sp*	Nambo Beach
IND-UHO 075	*Oscillatoria sp*	Nambo Beach
IND-UHO 076	*Chlorella sp*	Nambo Beach
IND-UHO 077	*Chlorella sp*	Nambo Beach

Screening of Newly Isolated Microalgae Suitable for Mass Cultivation in Outdoors in Hypersaline Media

Out of 77 isolates grown in liquid medium, there were about 8 isolates that showed good growth and did not stick to the culture vessels so that they remain in suspension to get expose to light and nutrients for optimum growth. These isolates were then screened for their ability to grow at high salinity (Figure 3).

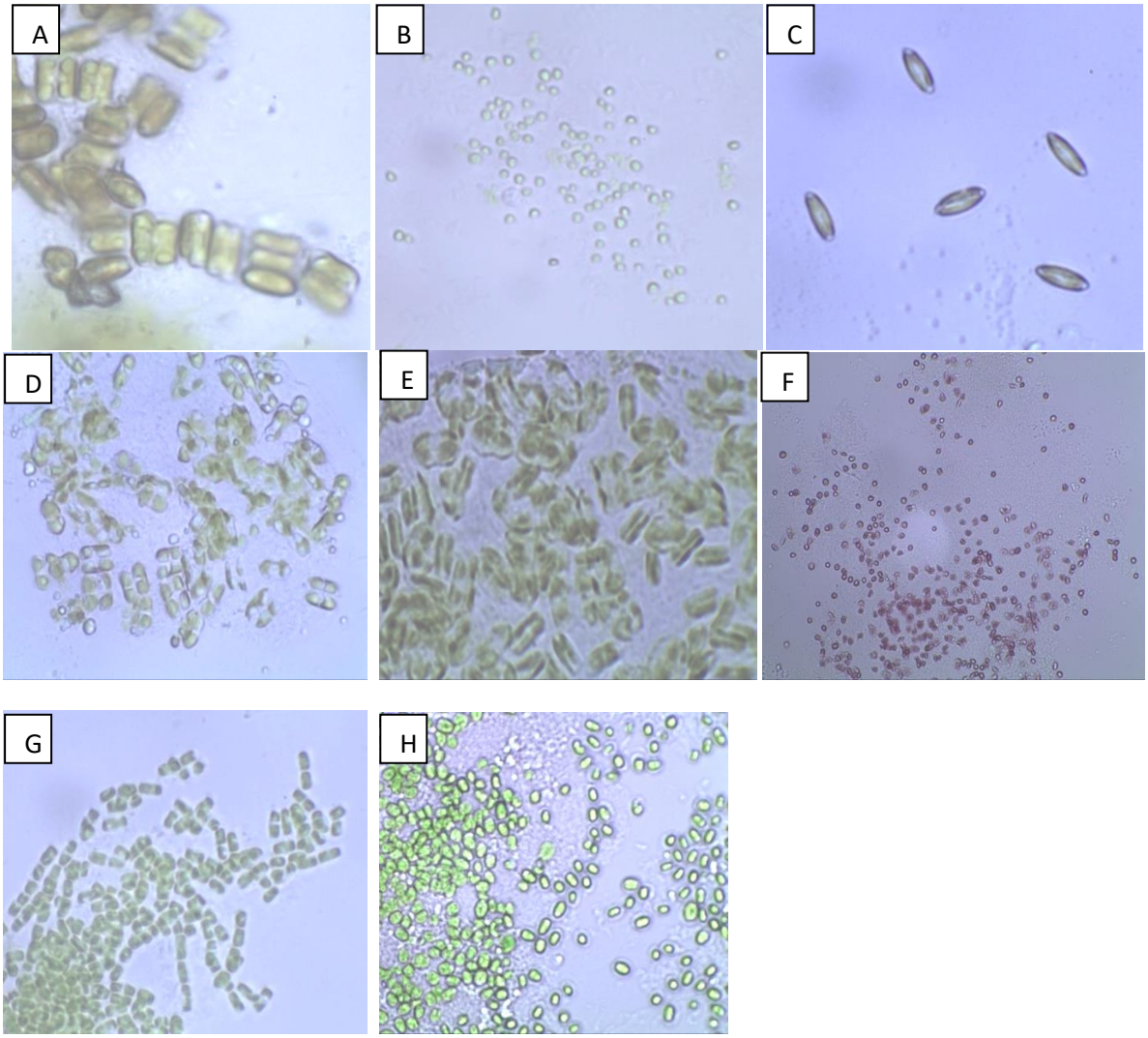


Figure 3. Photomicrograph (at 100x magnification) of Newly isolated microalgae potential for mass cultivation in outdoors: A.Isolate IND-UHO-002, B. Isolate IND-UHO-003, C. Isolate IND-UHO-017, D. Isolate IND-UHO-018, E. Isolate IND-UHO-019, F. Isolate IND-UHO-022, G. Isolate IND-UHO-029, H. Isolate IND-UHO-072

Out of eight isolates, three isolates namely isolate IND-UHO-029, IND-UHO-003 and IND-UHO-002 showed very good growth over wide ranges of salinity tested (3-5% NaCl) and showed no lag phase following inoculation indicating that they can adapt well at high salinity (Figure 4).

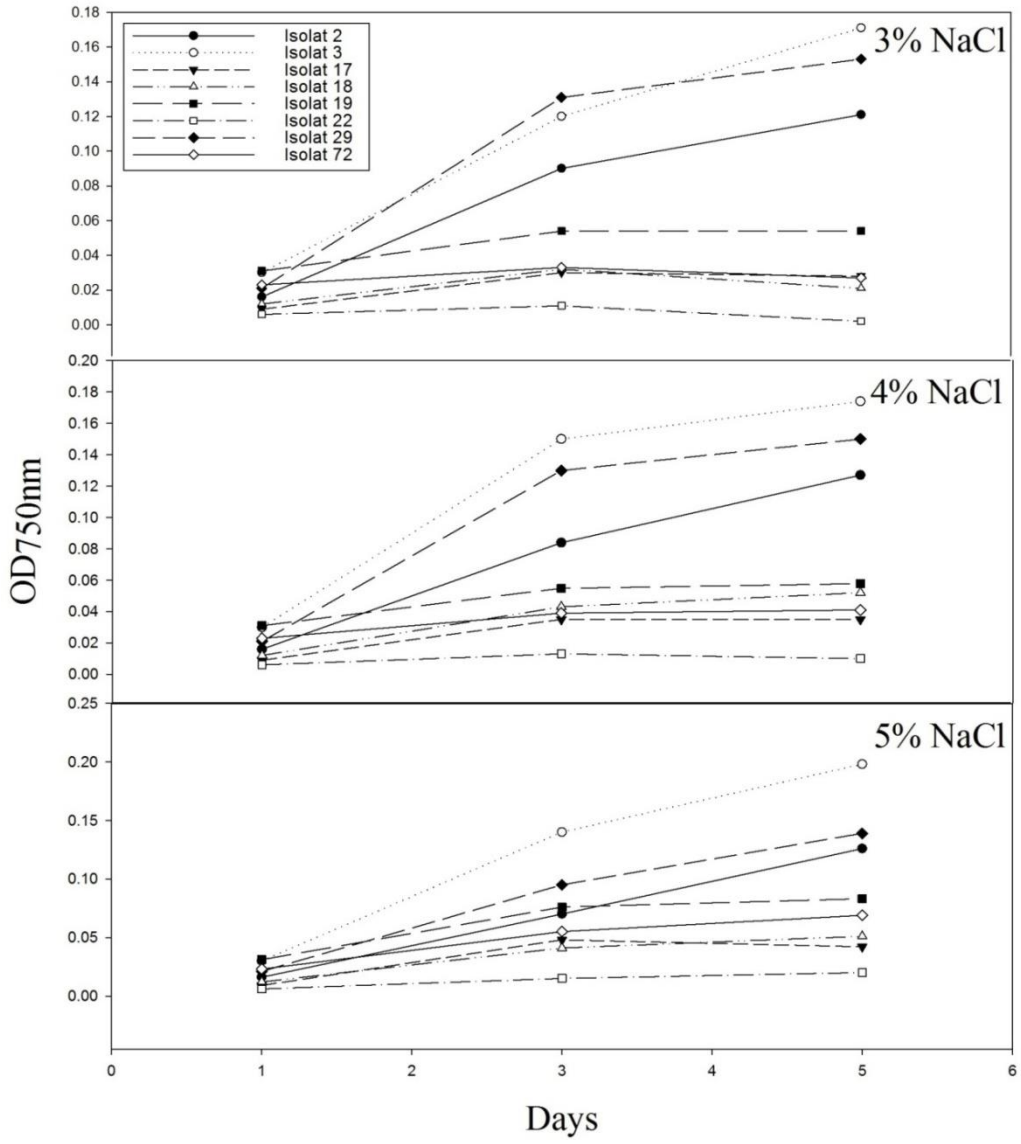


Figure 4. Growth curves of the newly isolated microalgae from Kendari Waters at different salinity

The specific growth rates of eight isolates under different salinity are shown in the Figure 5. The isolate IND-UHO-029 had the higest specific growth rate at salinity 3 and 4% NaCl followed by isolates IND-UHO-002 and IND-UHO-003. However, at 5%NaCl, the three isolate had about the same specific growth rate of about 0.74 - 0.77d^{-1}. Isolates IND-UHO-017 and IND-UHO-018 showed similar pattern in which the specific growth rate increased and reached maksimum at 4% salinity of about 0.68 and 0.64 d^{-1}, respectively before decreasing at 5% to about 0.64 and 0.61d^{-1}, respectively. It is interestingly to note that the specific growth rates of isolate IND-UHO 019, IND-UHO 022 and IND-UHO 72 increased as the salinity increase.

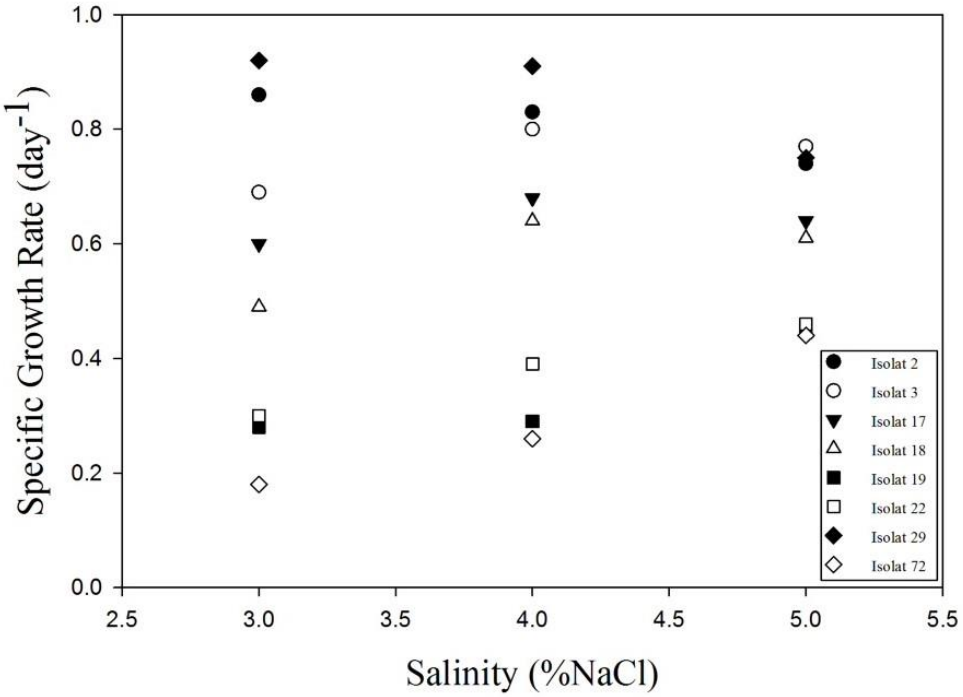


Figure 5 . Specific Growth Rates (day^{-1}) of the newly isolates microalgae from Kendari Waters under different salinity

Discussion

Isolation of microalgae using agar plating techniques is one of the most employed method of isolation due to its easy application and reliability in obtaining pure isolates. Although isolation of microalgae/phytoplankton is a time consuming and a labour intensive task, it is important for research and development of microalgae for any commercial applications. This study is the first in attempting to isolate local species microalgae from several coastal areas in Kendari Southeast Sulawesi, Indonesia. This study has been successfully generated hundreds of local species microalgae isolates which will be maintained and further explored for their potential commercial applications.

This study showed that isolation of microalgae straight away from plankton net samples using agar plating technique was unsuccesfull as not many colonies emerged on agar plates after 3-4 weeks of incubations. Also, the condition of the agar medium was not good as many holes formed on the agar and some of agar plates became watery. The possible explanation was due to the presence of zooplankton in the plankton net samples. The use of plankton net is not only for collecting microalgae/phytoplankton but also zooplankton including protozoan that feed on bacteria and phytoplankton. Therefore, when they were plated on agar, the zooplankton did grazing on bacteria/phytoplankton that grow on the surface of the agar forming holes and destroy the agar. However, when the enrichment samples were plated on agar plates, lots of colonies emerged and the condition of agar was still good (no holes and still in solid form). The possible explanation was because the samples collected manually contained less micro-organisms including microalgae and zooplankton. After enrichment with nutrients and under static condition, the microalgae started to grow and form colonies/aggregates. If the zooplanktons still exist in the samples, they will die as they are unable to feed on microalgae colonies/aggregates.

Newly isolated local species microalgae from coastal areas in Southeast Sulawesi are dominated by diatoms and filamentous cyanobacteria. The dominancy of diatoms isolates is not surprising as they are well known as the most abundance and diverse microalgae species found in almost all aquatic environments. Diatoms are unicellular microalgae that belong to Bacillariophyceae within the division of Heterokonthophyte (Falciatore and Bowler 2002) and considered to be the most important group of

phytoplankton responsible for almost 40% of marine primary productivity (Falkowski et al 1998). According to Scala and Bowler (2001), there are over 250 genera of diatoms with perhaps as many as 100,000 species which are more than three orders of magnitude in size of land plants and exhibit a remarkable variety of shapes. The diatoms can be easily distinguished based on their morphological characteristics especially their ability to generated highly ornamented silicious cell walls (frustules) constructed of two almost equal valves like a petri dish (Falciatore and Bowler 2002). Besides diatoms, another group of microalgae dominated the isolates is cyanobacteria. Cyanobacteria which is also known as blue-green algae are a diverse group of prokaryotic photosynthetic microorganisms that can grow rapidly due to their simple structures and growth requirements as well as efisicient use of light, CO_2 and other inorganic nutrients (Parmar et al 2011).

For commercial production of microalgae for any applications, microalgae should be able to grow at a large scale production system. Outdoor open pond systems are the most employed commercial production system of microalgae in the world (Borowitzka and Moheimani 2013a). For example, commercial production of *Dunaliella salina* for β-carotene production at Hutt Lagoon Australia (Borowitzka 2013b), *Arthrospira* by Earthrise Nutritionals, LLC (California, USA) and Hainan DIC Microalgae (China) and to produce astaxanthin from *Haematococcus pluvialis* by Cyanotech Co. (Hawaii, USA) and Parry Agro Industries Ltd (India) (Zittelli et al. 2013). However, not many microalgae are suitable for mass cultivation in outdoor open pond systems. There are several key criterias of microalgae suitable for large scale cultivation in outdoors including the ability of the microalgae to tolerate wide range of temperature and salinity ((Brennan and Owende 2010; Fon Sing et al. 2013; Indrayani 2017). In this study, we have successfully isolated and screened several microalgal strains that can grow well over a wide range of salinities and temperatures. There are three strains that show the highest growth rate at high salinity up to 5% NaCl (note that the seawater salinity ranging from 3-3.4% NaCl) namely *Diatoma* sp IND-UHO-002, *Chlorella* sp IND-UHO-003 and *Melosira* sp IND-UHO-029. The ability of microalgae to tolerate wide range of salinity and temperature are the most important criteria for the succesful of microalgal culture under outdoor conditions because outdoor cultures will be exposed to varying environmental conditions (Khatoon et al 2010). The salinity of the culture could decrease due to dilution of heavy rains or it could increase due to extensive evaporation of hot sunny days. Therefore, the microalgal species capable of growing well over wide range of salinity will not be affected by variation of the outdoor conditions and could sustain high productivy throughout the year. Microalgal species capable of growing at hypersaline environment will also be less prone to contamination as not many organisms could withstand high salt concentration and therefore monoalgal cultures could possibly be maintained for long periods (Borowitzka 2013a; Fon-Sing and Borowitzka 2016). Most importantly, microalgae capable to to grow at high salinity are more sustainable and economical to grow as they can utilize seawater which is free and plenty instead of use of precious and limited fresh water (Borowitzka and Moheimani 2013b; Resurreccion et al. 2012; Yang et al. 2011). Similarly, cultures will be exposed to daily and seasonal variation of temperatures. The microalgal strains were isolated from local environment and therefore the temperature optima for these algae generally reflect the temperature conditions found in their natural environments. For a tropical country like Indonesia, temperature is relatively constant with little fluctuation. The daily air temperatures range between 22-34^{o}C and that is the reason why we tested to grow the isolates at ambient room temperature (28-34^{o}C) and then selected the species that can withstand ambient temperature. As pointed out by Payer et al (1980), it is important to select microalgal strains that have optimum temperatures within the range of temperatures that will be encountered in outdoor.

Conclusion and Future Direction

Hundreds of newly isolated local species microalgae from coastal areas of Kendari Southeast Sulawesi Indonesia have been successfully established which are dominated by diatoms and filamentous cyanobacteria. Microalgae strains *Melosira* sp IND-UHO-029, *Chlorella* sp-IND-UHO-003 and *Diatoma* sp IND-UHO-002 are the most promising strain for mass cultivation in outdoor raceway ponds in saline-hypersaline media for commercial applications due to their ability to grow well in liquid medium, ability to grow at ambient temperature, ability to grow at high salinity and ability to remain in suspension. The next step of studies will be to determine their optimum growth conditions, to characterize their biochemical compositions (i.e lipid, protein, carbohydrate and carotenoid pigments) and to grow them under real condition in outdoor open pond systems. In the long run, the isolated species will be developed for commercial applications (i.e biodiesel and high value products).

Acknowledgements

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Authors:
Indrayani, Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Halu Oleo University, Kampus Bumi Tridharma Anduonohu, Kendari 93232, Southeast Sulawesi, Indonesia, e-mail: indrayani_tajudin@yahoo.com.au
Haslianti, Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Halu Oleo University, Kampus Bumi Tridharma Anduonohu, Kendari 93232, Southeast Sulawesi, Indonesia, e-mail:
Asi.haslianti@yahoo.co.id
Asriyana, Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Halu Oleo University, Kampus Bumi Tridharma Anduonohu, Kendari 93232, Southeast Sulawesi, Indonesia, e-mail: asriyana@uho.ac.id

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10. Paper 2 (Submitted to Jurnal Internasional, Biofuels/Indian Journal of Microbiology)

Effects of salinity on the growth and lipid production of A Newly Isolated marine microalga *Nannochloropsis* sp IND-UHO 003

Indrayani*, Haslianti, Asmariani

Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Halu Oleo University, Jl. HEA. Mokodompit, Kampus Bumi Tridharma Anduonohu, Kendari 93232, Southeast Sulawesi, Indonesia

Corresponding Author: indrayani_tajudin@yahoo.com.au

Abstract

Salinity affects growth and biochemical composition of microalgae and the ability of microalgae to tolerate wide range of salinity is one of the important criteria for succesful mass cultivation of microalgae in outdoor open pond systems. The aim of this study was to determine the effect of increasing salinity on the growth and lipid content of the newly isolated marine microalga *Nannochloropsis* sp IND-UHO 003. The strain was isolated from a shrimp pond in South Konawe, Southeast Sulawesi, Indoensia using agar plating technique (1% agar in f/2 medium). The microalgal strain was cultured in 500 mL Schott bottles containing 300 mL f/2 medium at increasing salinities from salinity 3, 4, 5, 6 and 7 % NaCl (in triplicates), under light intensity of about 100 μmol.photon.m^{-2}.s^{-1}, 12 hours light and 12 hours dark cycles and at room temperatures (25-34^{o}C). This study showed that the strain can grow well over the wide range of salinity tested (3-7% NaCl). The highest specific growth rate (0.76 d^{-1}) achieved at 3% salinity and the lowest (0.44 d^{-1}) obtained at 6% salinity. The highest biomass productivity was achieved at 4% salinity (0.8024 $g.L^{-1}.d^{-1}$) and the lowest at 3% salinity (0.3547 $g.L^{-1}.d^{-1}$). The cultures grown at 3% salinity had the highest lipid content and lipid productivity (27.21% AFDW and 0.1136 $g.L^{-1}.d^{-1}$, respectively). This study suggests that the *Nannochloropsis* sp IND-UHO-003 has a wide salinity tolerance (3-7% NaCl) and produce high biomass and lipid productivities at 4 and 3% salinity, respectively.

Keywords : Salinity, growth, lipid content, *Nannochloropsis* sp IND-UHO 003

Introduction

Microalgae are photosynthetic microorganisms including the prokaryotic cyanobacteria and eukaryotic microalgae that can be found in all ecosystems both aquatic and terrestrial (Richmond 2004; Mata et al. 2010). They are an extremely heterogeneous group of microorganisms which is not yet fully explored (Olaizola 2003; Borowitzka 2013). Microalgae are classified into one division of prokaryotic microalgae, the Cyanophyta (Cyanobacteria), and eight divisions of eukaryotic algae: the Glaucophyta, Rhodophyta, Cryptophyta, Heterokontophyta, Dinophyta, Haptophyta, Euglenophyta and Chlorophyta (Borowitzka 2012).

Microalgae have been suggested as biodiesel feedstock due to their ability to produce lipids that can be converted to biodisel (Chisti 2007; Mata et al. 2010; Parmar et al. 2011). Lipids produced by microalgae can be grouped into two categories, structural lipids (polar lipids) with a high content of polyunsaturated fatty acids (PUFAs) and storage lipids (non-polar lipids) mainly in the form of TAG made of predominantly saturated fatty acids and some unsaturated fatty acids (Sharma et al. 2012). Polar lipids (phospholipids) and sterols are important structural components of cell membranes which act as a selective permeable barrier for cells and organelles. These lipids maintain specific membrane functions, providing the matrix for a wide variety of metabolic processes and participate directly in membrane fusion events. In addition to a structural function, some polar lipids may act as key intermediates (or precursors of intermediates) in cell signalling pathways (e.g., inositol lipids, sphingolipids, oxidative products) and play a role in responding to changes in the environment. Of the non-polar lipids, TAGs are abundant storage products which can be easily catabolized to provide metabolic energy (Gurr et al. 2002).

Yields of microalgal lipids are higher than terrestrial crops. Depending on the lipid content , microalgae can produce about 58,700 – 136,900 L oil ha^{-1} $year^{-1}$ compared to that of soybean (636 L oil ha^{-1} $year^{-1}$), jatropha (741 L oil ha^{-1} $year^{-1}$), canola (974 L oil ha^{-1} $year^{-1}$) and palm oil (5366 L oil ha^{-1} $year^{-1}$) (Ahmad et al. 2011). Furthermore, microalgae are more sustainable to grow for lipid production due to their ability to grow on non-arable land and to utilise sea water so that they will not compete with food crops for habitats and for limited source of fresh water (Borowitzka and Moheimani 2013b). They also can use industrial flue gas as carbon source (Chisti 2007).

The lipid content in microalgae is species specific and it is influenced by environmental factors. Salinity is one of the important factor influencing the growth and biochemical composition of marine microalgae (Al-Hasan et al. 1987, 1990; Aizdaicher et al. 2010; Takagi et al. 2006; Rao et al. 2007; Zhila et al. 2011; Fon Sing et al. 2017; Indrayani et al. 2018). Salinity fluctuation is inevitable under outdoor conditions. When the microalgae species are cultured in outdoor open ponds, evaporation of the cultures in hot sunny days increases salt concentration in the medium. In contrast, dilution by rains decreases the salt concentration of the medium, emphasizing the importance of evaluating salinity tolerance of marine microalgae species. Therefore, the aim of this study was to determine the effect of salinity on the growth and lipid production of the newly isolated microalgal strain *Nannochloropsis* sp IND-UHO 003. This study could provide information about the salinity tolerance and the optimum salinity for growth and lipid production of the microalga *Nannochloropsis* sp IND-UHO 003.

Materials and Methods

Source of Algal Strain

The algal strain used in this study was *Nannochloropsis* sp IND-UHO 003 isolated from Kendari Waters, Southeast Sulawesi, Indonesia in June 2017. The strain was isolated using agar plating technique (Andersen and Kawachi 2005) in f/2 medium (Guillard and Ryther 1962). The strain is maintained in Microalgae Culture Collection at Faculty of Fisheries and Marine Science, Halu Oleo University, Kendari, Southeast Sulawesi, Indonesia.

Culture Condition

The *Nannochloropsis* cultures were grown in 500 mL conical flaks containing 300 mL of **f/2** medium at increasing salinity (3, 4, 5, 6 and 7% NaCl (w/v)). The cultures were initially grown at 3% salinity (seawater salinity) for 5 days in a batch mode to ensure cell growth before starting semi-continuous regime for over 2 weeks. The salinity of the cultures was increased gradually from 3 to 7% salinity during 3 weeks of semi-continuous mode. The cultures were incubated at ambient room temperature (26-32ºC), light: dark cycle (12 h:12 h) and irradiance (100 µmol photons.m-2.s-1) in triplicates. The cultures were bubbled with air to facilitate mixing the cultures. Cell counting was carried out every two days, whereas dry weight (DW), ash free dry weight (AFDW) and lipid were measured prior to harvesting.

Analytical methods

The growth of the cultures was monitored by counting the numbers of microalgae cells every two days using a Neubauer haemocytometer (Moheimani et al. 2013).

The specific growth rate (µ) was calculated using the following equation:

$$\mu = (Ln\left(\frac{N2}{N1}\right))/(t2 - t1)$$

Where *N1* and *N2* are the cell density at time 1 (*t1*) and 2 (*t2*).

For Dry weight (DW) determination, five mL of culture was filtered through pre-weighed and pre-combusted Whatman GF/C, 25 mm filter paper using a Millipore filter apparatus. The filters were removed from the Millipore filter apparatus, folded and patted dry with a paper towel. The filters were dried in an oven at 75ºC for 5 hours and then weighted (Moheimani et al. 2013). Dry weight (DW) was determined by the following equation:

$$Dry\ Weight\ (gram\ per\ liter) = weight\ of\ filter\ plus\ algae - weight\ of\ filter$$

The filters were then transferred to a furnace at 450ºC and ashed for 5 hours and weighted after cool. Organic dry weight (Ash-free dry weight) was calculated by the following equation:

$$Ash - Free\ Dry\ Weight\ \ (gram\ per\ liter) = DW - weight\ of\ ash$$

Biomass productivity was calculated by the equation :

$$Biomass\ productivity\ (gram\ per\ liter\ per\ day) = \ \mu\ x\ Yield\ (gram\ AFDW\ per\ Liter)$$

Total lipid determination was conducted by the method of Bligh and Dyer (1959) as modified by Kates and Volcani (1966). Five ml of cultures were filtered through Whatman

GF/C, 25 mm filter papers. The filters containing cells were put in 4 ml glass test tubes. The cells were then crushed with a glass rode until a smooth green paste of about 0.5 ml was obtained. 1 ml of the solvent mixture (methanol: chloroform: DI water in the ratio of 2:1:0.8 v/v/v) was added into the tubes, homogenized well with the glass rod and then transferred in a plastic centrifuge tube with screw cap. Another 1 ml of the solvent mixture was added into the glass tube to wash and clean all the remaining cells debris then transferred in the centrifuge tubes. Into the centrifuge tubes, 3.7 ml of the solvent mixture were added, tightly screwed to prevent solvent vapour loss then centrifuged at 3000 rpm for 20 minutes. After centrifugation, the supernatant was transferred to a 20 ml glass tube with screw cap. For the second extraction, 5.7 ml of the solvent mixture were added to pellet in the centrifuge tube, vortexed to resuspend the pellets then centrifuged again at 3000 rpm for 20 minutes. The supernatant was combined together in the 20 ml glass tube. Three ml of DI water and 3 ml of chloroform were added to the 20 ml tubes and mixed well by vortexing. The samples were then stored in the fridge undisturbed for 24 hours for complete seration. After incubation, two phases will be observed in the tubes, the top layer containing methanol and water and the bottom layer containing chloroform and the lipids. The upper layer was removed with a very fine pasteur pipette. About 6-8 drops of toluene were added to the chloroform layer, shaken by hand and then transferred to a dry and pre-weighed 10 ml vial. Any residual traces of water floating on the chloroform layer were removed using a very fine Pasteur pipette. The vials were then put under a stream of pure N2 gas with heating plates by 38^{o}C until complete dryness. After complete evaporation, the vials containing lipids were carefully weight using analytical balance. Weight of lipids is calculated by subtracting the weight of vials containing lipids with the weight of the vials. Lipid productivities were calculated using the following equation:

$$Lipid\ Productivity\ (gram\ per\ liter\ per\ day) = \mu\ x\ lipid\ yield$$

Statistical Analysis

Significant differences between treatments were analysed with a one-way analysis of variance (ANOVA). Normality Test (Shapiro-Wilk) was used to compare the means. All statistical analysis was performed using Sigma-Plot 14 Systat Software Inc., USA.

Results

Growth of the *Nannochloropsis* sp IND-UHO 003

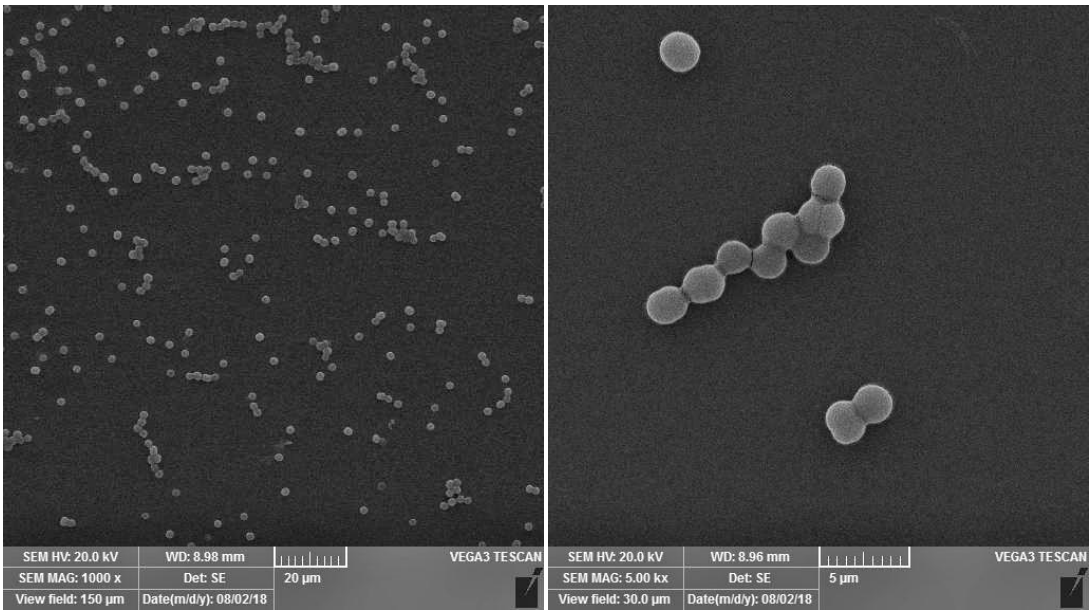

Figure 1. Scanning Electron Microscopy Images of the *Nannochloropsis* sp IND-UHO-003 at 1000x Maginification (left) and 10000x Magnification (right)

The growth of the *Nannochloropsis* for three weeks of culturing at increasing salinity from 3 to 7% NaCl under semi-continuous regime is shown in Figure 2. The alga was initially grown in a batch mode for 5 days at 3% salinity before initiating semi-continuous modes. The salinity of the cultures was increased by 1% after each harvesting (four days interval) until the salinity of the cultures reached 7% NaCl. The alga showed good growth over the wide range of salinity tested.

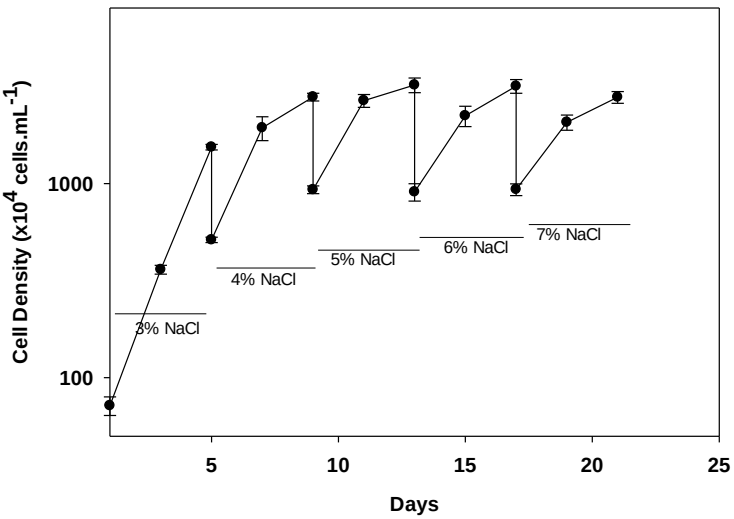

Figure 2. Growth of the *Nannochloropsis* sp IND-UHO 003 at increasing salinity under semi-continuous mode

The results showed that the specific growth rates of the *Nannochloropsis* decreased at increasing salinity. The highest specific growth rate (0.73±0.029 d^{-1}) was obtained at 3% salinity and the lowest specific growth rate (0.40±0.02 d^{-1}) was at 7% salinity (One Way ANOVA, $F_{(4,10)}$ =62.025, p<0.001) (Figure 3).

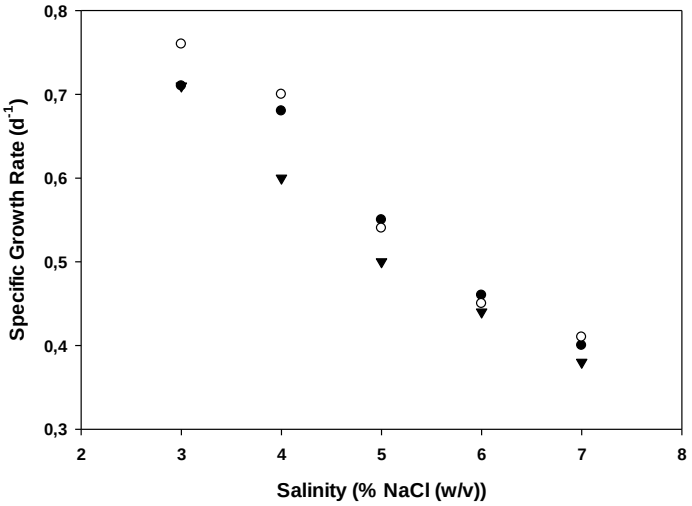

Figure 3. Specific growth rate (d^{-1}) of the *Nannochloropsis* sp. IND-UHO 003 at different salinity. Symbols represent the three replicate cultures

Biomass yield and productivity of the *Nannochloropsis* IND-UHO 003

Biomass yield of the *Nannohloropsis* was affected by the salinity tested. The highest biomass yield (1.10±0.1 $g.L^{-1}$) achieved at 4% salinity and the lowest (0.55±0.1 $g.L^{-1}$) obtained at 3% salinity (One Way ANOVA, $F_{(4,10)}$=21.243,p<0.001). Simiarly, the highest biomass productivity achieved at 4% salinity (0.73±0.12 $g.L^{-1}.d^{-1}$) and the lowest was at 3% salinity (0.39±0.07 $g.L^{-1}.d^{-1}$) (One Way ANOVA, $F_{(4,10)}$=12.690,p<0.001). It is important to note that both biomass yield and biomass productivity increased more than two fold when the salinity was increased from 3% to 4% NaCl (Figure 4).

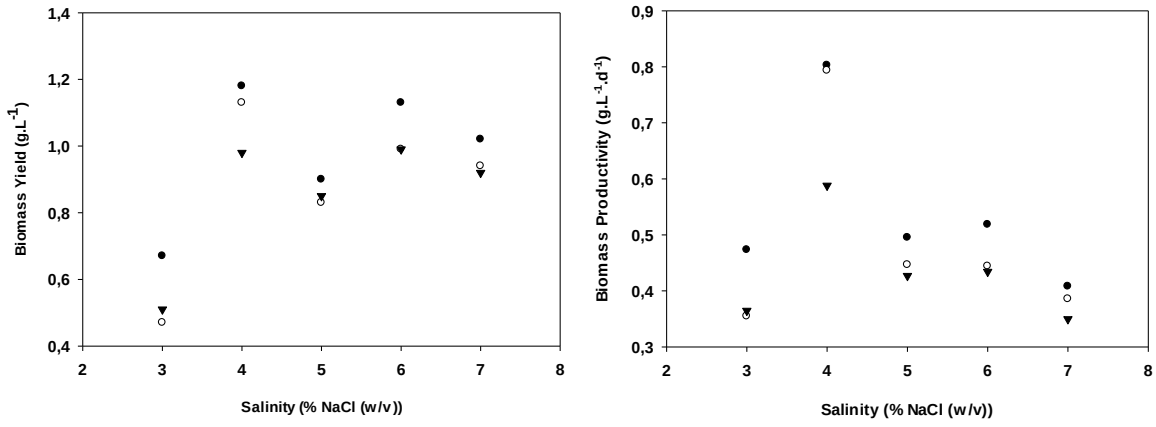

Figure 4. Biomass yield ($g.L^{-1}$) and Biomass productivity ($g.L^{-1}.d^{-1}$) of *Nannochloropsis* sp. IND-UHO 003 growing under different salinity. Symbols show the three replicate cultures.

Lipid of *Nannochloropsis* IND-UHO 003

The lipid yield of the Nannochloropsis ranged from 0.087±0.011 $g.L^{-1}$ to 0.167±0.011 $g.L^{-1}$. The highest lipid achieved at 5% salinity and the lowest occured at 4% salinity (One Way ANOVA, $F_{(4,10)}$=13.670, p<0.001) (Figure 5). The alga achieved its highest lipid content when grown at 3% salinity (25.05±1.9% AFDW) and the lowest lipid content achieved when the alga was grown at 4% salinity (8.11±0.75% AFDW) (One Way ANOVA, $F_{(4,10)}$=55.055, p<0.001) (Figure 5). The lipid productivity of the alga reached maksimum at 3% salinity (1.10± 0.011 $g.L^{-1}.d^{-1}$) and the lowest lipid productivity obtained at the highest salinity 7% NaCl (0.036±0.003 $g.L^{-1}.d^{-1}$) (One Way ANOVA, $F_{(4,10)}$=32.177, p<0.001).

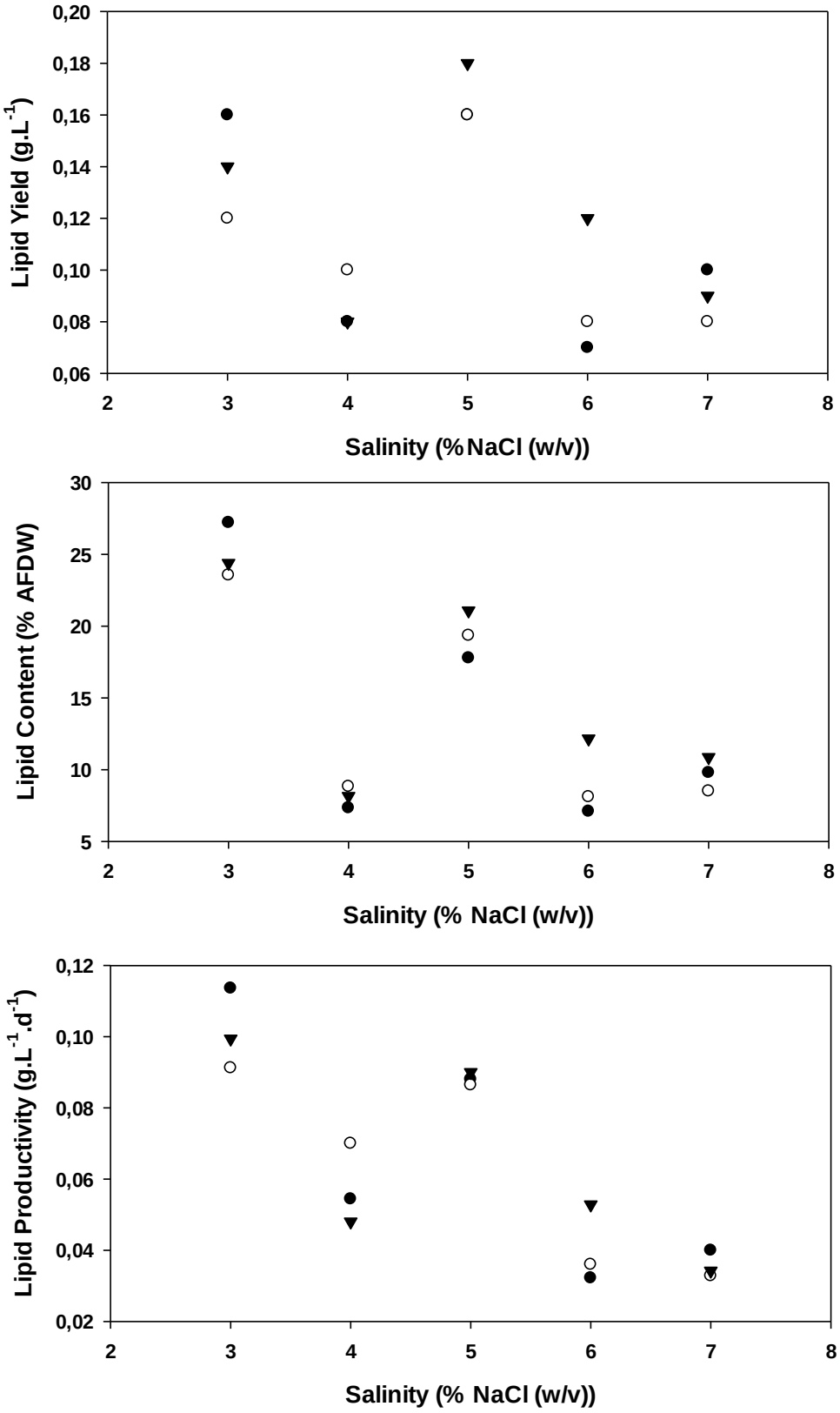

Figure 5. Lipid yield (g.L^{-1}), lipid content (% AFDW) and Lipid productivity (g.L^{-1}.d^{-1}) of *Nannochloropsis* sp. IND-UHO 003 at different salinity. Symbols show the three replicate cultures.

Discussion

The ability of microalgal strains to tolerate wide range of salinity is one of the important criteria for successful microalgal cultivation in outdoor open pond system. Therefore, it is important to determine the salinity tolerance of any microalgal species intending to be mass produced in outdoor open pond system for any commercial application. In this study, we found that the newly isolated *Nannochloropsis* sp IND-UHO 003 can grow very well over a wide range of salinity tested from 3 to 7% NaCl which is up to more than two fold of the seawater salinity. This is possibly the highest salinity tolerance reported in the literature for the genus Nannochloropsis (Eustigmatophyceae, Monodopsidaceae) (Renaud and Parry 1994; Hu and Gao 2006; Dispasmita Pal et al 2011; Gu et al. 2012).

In this study, we found that the highest specific growth rate of the *Nannochloropsis* sp IND-UHO 003 (0.73 ± 0.029 d^{-1}) was achieved at 3% NaCl and the lowest (0.40 ± 0.02 d^{-1}) was obtained at 7% salinity. Compared to the SGR reported on the genus Nannochloropsis, the SGR reported in this study was much higher than others. For example, the highest specific growth rate of *Nannochloropsis oculata* was about 0.282 ± 0.017 d^{-1} obtained at salinity 35 g/L or 3,5% NaCl (Gu et al. 2012). Renaud and Parry (1994) reported the optimum salinity for the growth of the *N. oculata* was 22 to 25 g/L (2.2 to 2.5% NaCl). The optimum salinity for growth of the *Nannochloropsis* sp IND-UHO 003 is 3% NaCl. This is not surprising because the strain bloomed in the shrimp pond where the strain was originally isolated (note that the salinity of the pond was 2.9% NaCl).

Microalgal growth rate, lipid content and lipid productivity are the main criterias in determining the potential of microalgal strains for biodiesel feedstock (Gong and Jiang 2011).

In conclusion, this study suggests that the microalga strain Nannochloropsis sp IND-UHO 003 has a wide range of salinity tolerance from 3% to 6% NaCl concentration. The highest biomass and lipid productivites of the alga were achieved at 4% NaCl and 3% NaCl, respectively. Research on this strain is continuing to determine other limits to growth factors to further enhance biomass and lipid productivities for biodiesel and high value product production (i.e DHA and EPA contents).

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