UTILIZATION OF INVASIVE ALGAL BIOMASS FOR BIOETHANOL PRODUCTION AND THE DYNAMICS OF PLANKTONIC FUNGI IN THE WEST PACIFIC

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ABSTRACT

Algae represent the most promising feedstock for biomass derived biofuel production. Certain invasive algae in Hawaii can form dense biomass and are potential feedstocks for bioethanol production. In this study, the biomass from the invasive algae *Gracilaria salicornia* was used as feedstock for ethanol production using the ethanologenic strain *Escherichia coli* KO11. The algal hydrolysates were successfully utilized in a two-stage saccharification and fermentation platform, showing no inhibition of its bacterial fermenting ability, and producing 79.1 g ethanol from one kilogram of dry algal mass. Algae contain large quantities of species-dependent polysaccharides that cannot be readily metabolized by current ethanologenic bacteria. To fully explore the potential of microbial conversion of algal biomass and increase the systematic efficiency for ethanol production, culture-dependent and independent methods were applied to identify bacterial candidates fulfilling these purposes. The microbial communities profile associated with selected native and invasive algae were determined, which supplied valuable information in searching for candidates for polysaccharides utilization. Furthermore, microbes that can facilitate consolidated bioprocessing (CBP)—a process that can potentially optimize the systematic efficiency of biomass derived ethanol production—are isolated from various sources. Two bacteria FNP1 and TF2 showed great potential in further engineering for CBP platform development. Collectively, this study supplied valuable information in developing an efficient bioethanol production platform using invasive algal biomass.

The dynamics of planktonic fungi in the west Pacific was investigated in part II of the dissertation. This study revealed that planktonic fungi are molecularly diverse and the
fungal distribution was related to major phytoplankton taxa and various nutrients including nitrate, nitrite, orthophosphate and silicic acid. Over 400 fungal phylotypes were recovered and nearly half of them grouped into two major novel lineages. Ascomycota and Basidiomycota were found to be dominant groups at majority of the investigated stations. These results suggest that planktonic fungi are an integral component of the marine microbial community and should be included in future marine microbial ecosystem models.
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PART I

BIOETHANOL PRODUCTION FROM INVASIVE ALGAL BIOMASS AND THE
CULTURE-DEPENDENT AND INDEPENDENT PROSPECTING OF BACTERIA
FOR BIOFUEL PRODUCTION
Chapter 1. General introduction

Increasing efforts are being dedicated in the alternative fuel search due to the volatile oil price and unsustainable fossil fuel consumption. Although it has yet to be economically competitive, biofuel merits full attention because it is fully renewable, can be developed using diverse strategies, and has environmental benefits [1, 2].

Platforms of current biofuel production

Biofuel production strives for the efficient extraction of solar energy collected by photosynthetic cells to produce desired biofuel products. Versatile metabolic pathways in photosynthetic bacteria, algae and plant cells present diverse platforms for developing biofuel products.

Photosynthetic bacteria can be incorporated into biofuel production in various ways. One approach using the cyanobacterium *Synechococcus elongatus* employed a heterologous isobutanol pathway, thus equipping the bacterium with the ability to directly convert CO$_2$ to isobutanol, a valuable four-carbon alcohol [3]. At Venter Institute, a novel developmental strategy is currently used to create a hybrid microbe combining features of a cyanobacterium and a photosynthetic bacterium in order to produce hydrogen from water with the presence of oxygen [4]. The discussion on the potential application of microalgae in biofuel production can be traced back to more than 50 years ago [5, 6]. The endeavor to develop high-oil-producing and fast-growing microalgal species was fully demonstrated in the aquatic species program (ASP), an over two-decade long algae screening program supported by the US Department of Energy [6]. The oil (lipids or triglycerides) produced in microalgae cells can be directly extracted and further converted to biodiesel by transesterification [7, 8]. Metabolic engineering of algae cells
also enabled the production of other types of fuels including hydrogen, ethanol and crude oil resembling long-chain hydrocarbons [9, 10]. A realized platform of microalgae fuel production could be revolutionary, provided that several challenges are readily overcome including obtaining economically competitive microalgae species, developing efficient genetic manipulation tools and lowering the cost of culturing and harvesting algae cells [10, 11].

A third platform for biofuel production, which converts biomass sugars into desired biofuel products via microbial fermentation, represents the most promising alternative by far. Ethanol constitutes 99% of total biofuel consumption in the US with increasing demands as a major fuel additive [12]. First generation bioethanol production primarily uses corn starch as feedstock. Competition with the food industry, however, hinders the long-term development of this approach. The abundant and renewable cellulosic biomass renders a valuable feedstock for the second generation bioethanol production [13]. However, the biggest challenge posed in the current cellulosic ethanol production is to develop a cost-effective strategy to reduce biomass recalcitrance and improve hydrolysis efficiency [14, 15]. Nevertheless, the genetic engineering of energy crops for more affordable feedstock [16], crop cultivation in marginal land [17] and net energy gain from cellulosic ethanol production [18] all contribute to the increasing efforts devoted in the development of a realized cellulosic ethanol production. Applying algal (microalgae and macroalgae) biomass for ethanol production, sometimes referred as the third generation bioethanol production, has been widely acknowledged [19]. This approach favors algae as the feedstock where the algal biomass is either readily available or easily obtained. The use of algae is reinforced by its features: high photosynthetic
efficiency, cultivable in diverse habitats (e.g., seawater, blackish water, saline water and wastewater), and efficient recycling of carbon dioxide [20, 21]. In Hawaii, the tropical marine environment sustains a variety of marine macroalgae including both native and invasive species. About a dozen alien algal species introduced to Hawaii have become invasive [22]. Although the ecological and financial impact resulting from invasive algae can be detrimental (introduced in chapter 3), the amount of macroalgae biomass obtained from invasive algal management could be enormous [23]. In the meantime, the highly successful colonization and dense biomass formation of certain invasive algal species could be valuable in terms of accumulating large quantities of biomass feedstock, provided that strict management policies are applied to avoid their adverse environmental impact. The invasive algae *Gracilaria salicornia* was documented for their successful colonization and dense biomass formation ability in Hawai’i coast [23, 24]. This study thus explored the possibility of using the ‘waste’ macroalgae *Gracilaria salicornia* as feedstock to produce bioethanol.

**Two-stage vs. consolidated strategy**

The traditional approach for biomass based ethanol production is divided into two separate stages: saccharification and fermentation. A pretreatment step is usually required to reduce the biomass recalcitrance and make the polysaccharides more accessible for further chemical or enzymatic hydrolysis [25]. As a matter of fact, the fermentability of the hydrolysate is heavily dependent on the efficiency of the pretreatment step [26]. Dilute sulfuric acid pretreatment has a long history of application and is still broadly used in the current industrial process (introduced in chapter 2) [27, 28]. The hazards involved with handling concentrated acid and the complexities of recycling it [29], however,
encouraged the development of more efficient methods using less hazardous and more tractable cellulose solvents [30]. Binder and Raines reported their method of using room-temperature ionic liquids as cellulose solvents efficiently yielded nearly 90% of the glucose from cellulose and 70-80% of sugars from untreated corn stover [29]. This approach was successfully applied in other studies [2, 31], and could be a viable solution for future scalable biorefinery from crude biomass. Ammonia Fiber Expansion (AFEX), another interesting approach, not only produces a highly fermentable hydrolysate but can also enrich the nutrient content of AFEX-treated biomass [26, 32, 33]. The enzymatic hydrolysis of pretreated biomass using various glycoside hydrolases can further increase the concentration of fermentable sugars in the hydrolysates, but has one of the biggest raw material costs [34]. Consolidated bioprocessing (CBP), a process combining saccharification and fermentation stage into a single organism, is being proposed as a potential solution to this challenge [35]. A microbe that can facilitate consolidated bioprocessing features anaerobically saccharification and is able to ferment simple sugars into the final desired biofuel products. These microbes are not readily available and are being developed in various ways (further introduced in chapter 4) [2, 36-38].

**Fermentable sugars in the macroalgal biomass**

Many marine algae have rich carbohydrate contents, including cell wall polysaccharides (cellulose, hemicelluloses, xylan and mannan), intercellular polysaccharides (sulfated glucuronoxylorhamnan, algine, agar and carrageenan), and storage polysaccharides (amino pectin, laminaran and floridean starch) [39]. Unlike plants, algae have little or no lignin and is structured with cellulose Iα instead of cellulose Iβ as the predominant cell wall polysaccharide [19, 40]. These features collectively make
algal biomass less rigid to the chemical or enzymatic hydrolysis, favoring its selection as the biomass feedstock rather than lignocellulosic biomass. Glucose released from starch or cellulose hydrolysis represents the most direct and preferred sugar stock for microbial fermentation. However, hemicelluloses and other algal species-dependent heteropolysaccharides constitute a major portion of the polysaccharide pool in the algal biomass [41]. For example, the red algae Gelidium amansii is found to be rich in galactan, the polymer formed by D- and L-galactose derivatives [19, 41, 42]. The brown algae, such as Laminaria sp. and Saccorhiza sp., are usually abundant in laminarin and mannitol [19, 43]. The real challenge for algal biomass derived bioethanol production is the microbial conversion of various compositions of monosaccharides hydrolyzed from galactan, alginate, carageenan, agar etc. in the algal hydrolysates.

The thriving effort dedicated to the microbial development of mixed sugar fermentation is prompted by the fact that no microbe in its natural state can ferment all simple sugars [44]. Wild type microorganisms like Saccharomyces cerevisiae or Clostridium sp. can only metabolize one or a few simple sugar sources (glucose or xylose) in algal biomass hydrolysates [21]. To increase the efficacy of ethanol production, various microbial strains have been genetically modified to eliminate redundant metabolic pathways, and to expand their sugar consumption ability and by-products tolerance [45, 46]. The xylose-catabolizing pathway has been successfully introduced into natural bacteria to ferment both hexoses and pentoses [47, 48]. As far as this is concerned, the engineering of various metabolic pathways like glycolytic, pentose utilization, and ethanologenic is well advanced [49, 50]. The state of the art rather resides in the configuration of multiple sugar transporters and their regulation given the fact of
diverse sugar contents in algal hydrolysates [51, 52]. Recently, an exciting development towards this goal resulted in an engineered ethanologenic *E. coli* strain with the ability to transport and metabolize alginate, an abundant polysaccharide in brown algae [53]. Ultimately, the clarification of sugar regulation will help gain insight into the development of simultaneous mixed sugar fermentation, and would be a major advance towards a realized industrial scale biorefinery [52].

**Objectives of the study**

In this study, the potentials of bioethanol production using biomass from invasive algae *G. salicornia* was explored. The bacterium *E. coli* KO11 was used in this study for its ability to ferment mixed sugars including both hexose and pentose [47, 54]. This part of dissertation is divided into three chapters that fulfill the following objectives:

1) To explore the fermentability of hydrolysates from the invasive algae *G. salicornia* by the ethanologenic *E. coli* KO11 (Chapter 2).

2) Applying culture-independent methods to identify bacterial candidates with the ability of metabolizing algae-dependent sugar stock (Chapter 3).

The efficiency of bioethanol production from algal biomass hydrolysates is largely dependent on the ability of microbes to metabolize mixed sugars. The microbial community associated with algae is thus a valuable source for searching these microbes. The microbial communities associated with two pairs of Hawaiian native and invasive algae were investigated and their associated microbial community profile was constructed to gain insight into potential algae-dependent sugar fermenters.

3) Apply culture-dependent methods to prospect for microbes that can facilitate the consolidated bioprocessing (CBP) (Chapter 4).
CBP represents the most promising approach towards an achieved platform for biomass derived biofuel production, albeit there is no fully developed microbe currently available for this purpose. The bioprospecting of microbes that can facilitate CBP can be found in chapter 4.
Chapter 2. Two-stage hydrolysis of invasive algal feedstock for ethanol fermentation

Abstract

The overall goal of this work was to develop a saccharification method for the production of third generation bioethanol using feedstock of the invasive marine macroalga *Gracilaria salicornia*. Under optimum conditions (120°C and 2% sulfuric acid for 30 min), dilute acid hydrolysis of the homogenized invasive plants yielded a low concentration of glucose (4.1 mM or 4.3 g glucose/kg fresh algal biomass). However, two-stage hydrolysis of the homogenates (combination of dilute acid hydrolysis with enzymatic hydrolysis) produced 13.8 g of glucose from one kilogram fresh algal feedstock. Batch fermentation analysis produced 79.1 g EtOH from one kilogram of dried invasive algal feedstock using the ethanologenic strain *Escherichia coli* KO11. Furthermore, ethanol production kinetics indicated that the invasive algal feedstock contained different types of sugars, including C5-sugar. This study represents the first report of the third generation biofuel production from invasive macroalgae, suggesting that there is great potential for the production of renewable energy using marine invasive biomass.
Introduction

Like plants, many algal species have rigid cellulose-based cell walls and accumulate starch as their main carbohydrate storage compound and cell wall structure, which contains an astonishingly diverse range of simple and complex carbohydrates \([55, 56]\). Some of marine algal species contain up to 70% of polysaccharides, \textit{i.e.}, cell wall polysaccharides (cellulose, hemicelluloses, xylan and mannan), intercellular polysaccharides (sulfated glucuronoxylorhamnan, algine, agar and carrageenin), and storage polysaccharides (amino pectin, laminaran and floridean starch) \([39]\). Both intercellular and cell wall polysaccharides can be fermented to produce ethanol. Additionally, algal feedstocks have several advantages over other types of feedstocks. These include high area productivity, no competition with conventional agriculture for land, utilization of different water sources (e.g., seawater, blackish water, saline water and wastewater), recycling of carbon dioxide, and compatibility with integrated production of fuels and co-products within biorefineries. Hence, algal feedstocks are considered one of the most promising non-food feedstocks for biofuels \([57]\). Previous studies of algal biofuel production have largely focused on microalgae \([58]\). Mannitol extracted from the brown seaweed \textit{Laminaria hyperborean} has been used as a substrate for ethanol production by \textit{Zymobacter palmae} with a yield of 0.38 g ethanol per gram mannitol \([59]\). A conceivable biorefinery production process of third generation ethanol using the seaweeds \textit{Euchema} spp. has been proposed \([55]\). However, detailed studies of macroalgal feedstock hydrolysis for ethanol fermentation are rare. Particularly, production of third generation biofuel from invasive macroaglae has not been reported.
Ethanol was successfully implemented as the chief component of automotive fuel in Brazil nearly three decades ago. Currently, ethanol is commonly used in transportation fuel, with blends up to 15% with gasoline. It constitutes 99% of total biofuel consumption in the USA. Regardless of its low energy content in comparison with gasoline, ethanol has many advantages as an alternative energy, including reduction of pollutant emissions and production from environmental wastes [12]. Corn (Zea mays) grain and other cereals like sorghum (Sorghum bicolor) constitute the primary feedstock for ethanol production in the USA and other countries [18]. However, these feedstocks are major food sources and ethanol production from these grains can seriously impact food prices and the economic viability of ethanol production. Thus, ethanol from nonfood feedstock is a key factor for the economic viability of its production. Among various nonfood feedstocks, algae (macro- and microalgae) have a higher photosynthetic efficiency and have long been considered as one of the most promising non-food feedstock for biofuel (i.e., ethanol and diesel) production [57, 60].

Marine macroalgae contribute significantly to the global primary production and play critical roles in the stability and function of marine ecosystems [61, 62]. However, macroalgae can also become invasive in a newly introduced environment, and then have profound adverse ecological impacts including the alteration of ecosystem structure, reduction of indigenous biodiversity, and economic losses [23, 63-65]. There are about two dozen alien algal species in Hawaii with half of them being reported as invasive [22]. Management of invasive algae in Kihei (Maui) alone costs the state of Hawaii $20 million annually [23]. Invasive algae are also found in other coastal areas of the state, including Waikiki and Kaneohe Bay (Oahu) and southern Molokai [63]. Therefore, the
statewide economic impact of invasive algae is greater than $20 million annually. During blooming time, some of these species (e.g., *Gracilaria salicornia*) are washed up on beaches in large quantities. The large piles of decomposing biomass draw complaints from residents and drive tourists away [66]. The invasive species *G. salicornia* is commonly found in Hawaii and has been reported to kill corals in Waikiki and Kaneohe [23, 63]. Currently, physical removal has been the only reported efficient method for management of invasive algae. Over twenty tons of biomass for the invasive species *G. salicornia* were removed from the area of the War Memorial Natatorium in Waikiki (Oahu) at a clean-up event [23]. For these reasons the management of large quantities of invasive algae biomass presents a great environmental challenge.

In this study, we took advantage of the high content of cellulosic biomass of the invasive species *G. salicornia* and demonstrated the possibility of producing ethanol from the invasive algal feedstocks. It represents the first report of ethanol production from invasive macroalgae.

**Materials and methods**

*Algae specimen collection*

Algal plants of the invasive species *Gracilaria salicornia* were collected on a reef in the subtidal zone near Waikiki Beach, Oahu Island. Specimens were directly transferred to zip lock plastic bags. Latex gloves were worn during collection. The samples were transported to the laboratory within 1 hour and processed immediately for homogenization.

* Dilute acid hydrolysis of algal biomass*

Algal plants were rinsed briefly with sterile water and dried with paper towers. The washed algal plants were homogenized in a homogenizer. Separation of agar and
cellulosic fibrin used the method described by Guerin and Bird [67]. The resulting agar and cellulosic fibrin fractions were individually hydrolyzed using the dilute sulfuric acid hydrolysis [68]. To optimize acid concentration, each part of the two fractions was mixed with sulfuric acid with final concentrations of 0.5%, 1%, 2.5%, and 5% (v/v) and hydrolyzed at room temperature for 30 min. To optimize hydrolysis temperature, agar and cellulosic fractions containing 2% and 2.5% of sulfuric acid, respectively, were autoclaved at different temperatures (105, 115, 125 and 128°C) for 30 min. The resulting hydrolysate was centrifuged at 6,000 × g for 10 min, and the resulting supernatant containing mono-sugars was collected and neutralized with NaOH to pH 7. Glucose content of the hydrolysates was determined using Glucose Assay Kit (Sigma) and then used to choose the optimal hydrolysis condition. All treatments were done in three replicates.

**Enzymatic hydrolysis of algae biomass**

For enzymatic hydrolysis, the homogenate of the invasive algal plants containing 2% sulfuric acid was hydrolyzed at 120°C for 30 min. The resulting hydrolysate was neutralized to pH 5 with NaOH and mixed with NaOAc/AcOH buffer (0.05 M, pH5) at the volume ratio of 1:1. 1000 ml of the buffered hydrolysate was incubated with 5g of cellulase (MP Biomedicals, LLC) at 40°C. At certain time points, 1 ml of the enzyme mixture was collected and centrifuged at 14,000 × g for 10 min. The resulting supernatant containing mono-sugars was filtered through Whatman filter paper, 2.0-μm and 0.2-μm polycarbonate filters sequentially. Glucose was then detected by RID detector equipped onto the high-performance liquid chromatography (HPLC) (Shimadzu) [69]. A 20 µl filtrate was loaded and ran through a BioRad HPX-87H ion exchange column (300 mm ×
7.8 mm) (60°C) at 0.3 ml/min with an isocratic mobile phase of 0.008 mM sulfuric acid. The calibration curve for glucose concentration was plotted using various glucose concentrations (3.75, 7.5, 15, 30 and 60 mM) against their glucose peak areas. The calibration curve was set up once and used for all measurements.

**Fermentation of algal hydrolysates**

Neidhardt MOPS defined minimal broth with supplements was used for anaerobic batch fermentation following the method described by Mathews et al [70]. In brief, the batch fermentation was carried out in a 4-part assembly 500 ml capacity spinner flask (Bellco Blass), with a total volume of 400 ml of minimal Neidhardt MOPS defined broth with algal hydrolysate as the carbon source. 400 ml of the fermentation broth was made from 300 ml of algal hydrolysate, 1 × MOPS minimal, 1.32 mM of K$_2$HPO$_4$, 1 × ACGU solution (TEKNOVA), 1 × supplement EZ (TEKNOVA), 50 µg/ml chloramphenicol and 25.6 ml sterile water. The media inside the spinner flask and the space in the attached gas trap was sparged with nitrogen gas for 20 min to remove oxygen. All fermentations were done at 30°C using a circulating water bath.

For the fermentative production of ethanol, an *Escherichia coli* KO11 strain [71] was inoculated into 100 ml of LB broth supplemented with 2% xylose and incubated overnight at 37°C. On the day of fermentation, 5 ml of the resulting bacterial culture was reinoculated into 100 ml LB broth supplemented with 2% xylose in the morning and incubated at 37°C until the OD$_{600}$ reached 1.00. Bacterial cells were then collected by centrifuging at 5,000 xg for 10 minutes and washed twice with sterile distilled water. Finally, the washed bacterial cells were suspended in 25 ml of fermentation media and injected into fermentation system.
For ethanol and other analysis, 1 ml of fermentative broth was drawn from the fermentation system at time points of 0, 2, 4, 6, 9, 12, 15, 17, 20, 23, 26, 30, 34, 42, 45 and 50 hour. The fermentative broth was centrifuged at 16,000 ×g for 1 minute, and the supernatant was filtered through a 0.22-µm of polycarbonate membrane. The filtrate was used to determine glucose and ethanol concentrations by HPLC as described above [70]. The calibration curve for ethanol measurement was plotted using four standard ethanol concentrations (6.25, 12.5, 25 and 50 mM) against their peak areas. Experiments were conducted in triplicate for fermentation analysis. Neidhardt MOPS defined broth supplemented with 30 mM of glucose was used as a positive control.

**Results**

*Optimizing dilute acid hydrolysis of algal biomass*

Dilute acids break down the cellulose and hemicellulose polymers in cellulosic biomass to release individual sugars that can be fermented into ethanol [72, 73]. Kinetic studies on the dilute acid hydrolysis of various cellulosic materials indicated that the hydrolysis kinetic parameters are strongly dependent on the substrate and acid concentration, temperature and reaction time [73, 74]. However, macroalgal plants have rarely been investigated for saccharification using the dilute acid hydrolysis. To optimize the conditions for the hydrolysis, the homogenates resulting from the invasive algal plants were heated to 100°C and cooled down to room temperature. The agar fraction was separated from cellulosic one. Individual fractions were used to optimize their conditions for saccharification using dilute acid hydrolysis. Of the four tested sulfuric acid concentrations, dilute acid hydrolysis using 2.5% sulfuric acid yielded the highest glucose concentration from the cellulosic fraction, and hydrolysis with 2% sulfuric acid
gave the best results from the agar fraction (Fig. 1). At their optimal sulfuric acid conditions, the hydrolysis at 125°C gave the highest glucose concentration from both agar and cellulosic fraction of algal biomass (Fig. 2). To reduce the cost of separating agar from cellulose fraction, the homogenates of the invasive algal plants were hydrolyzed at the level of 2% sulfuric acid and 120°C for 30 min. Glucose concentration in the resulting hydrolysates contained approximately 4.1 mM (i.e., 4.3 g glucose/kg wet algal biomass) and failed to yield detectable ethanol in the batched fermentation (data not shown).

**Two-stage hydrolysis of invasive algal biomass**

To improve the saccharification efficiency of the invasive algal plants, the homogenate containing both agar and cellulose components was subjected to the dilute acid hydrolysis containing 2% sulfuric acid at 120°C for 30 min. The resulting hydrolysates were hydrolyzed using cellulose as described previously. Glucose concentration in the hydrolysates resulted from the two-stage hydrolysis reached 15.1 mM at 4-h incubation and continued to rise up to 16.6 mM after 26-h incubation (Fig. 3). Overall, after 4-h incubation, further enzymatic hydrolysis did not significantly increase glucose concentration. Therefore, 4-hour incubation was used for enzymatic hydrolysis in this study.

**Ethanol production from algal hydrolysates**

The ethanologenic strain *E. coli* KO11 [71] was used in batch fermentation for the production of ethanol with the algal hydrolysate as the sole carbon source. In media containing the algal hydrolysate, 13.8 mM glucose was completely consumed within 18 h, with little variation in all 3 replicate fermentation experiments (Fig. 4). The ethanol
concentration reached approximate 28 mM when glucose was used up. However, ethanol concentration continued to increase up to 34 mM with further incubation. The continued increase in ethanol concentration clearly indicated that other non-glucose sugars occurred in the algal hydrolysate and were fermented by *E. coli* KO11 cells into ethanol (Fig. S1). Ethanol concentration reached the maximum (34 mM) at 30-h incubation, which indicates that extended fermentation did not increase ethanol concentration in the fermentor (Fig. 4). The described process using the invasive algal plants feedstock, two-stage hydrolysis, and *E. coli* KO11 as the ethanologenic strains in fermentation was able to achieve 79.1 g EtOH per kg of dried invasive algal plants. In the positive control, 30 mM of glucose supplemented in Neidhardt MOPS defined minimal media as the carbon source was completely consumed at 18-h fermentation and yielded the highest ethanol concentration of 44 mM (Fig. 5).

**Discussion**

Algal feedstocks are one of the most promising non-food feedstocks for biofuels [57]. In this manuscript, we developed a two-stage hydrolysis method for the saccharification of invasive marine algal plants and used *E. coli* KO11 as the ethanogenic strain to successfully ferment invasive algal monosaccharides into ethanol. This is the first report on the production of ethanol from marine invasive species. It provides useful techniques to produce biofuel from marine biomasses, particularly from those that are an environmental hazard. Given the fast-growing nature of the invasive algae, the resulting methods and technology can be used to produce biofuel from marine biomass collected in Hawaii or any other coastal region of the world’s ocean. This study contributes to the pursuit of viable renewable energy production from marine bioresources and also methods to reduce CO₂ emissions.
First generation biofuels refer to fuels derived from sources like corn starch, sugar cane or sugar beet, animal and vegetable fats [75]. The major concern related to the first generation biofuels is that they compete for arable lands with food crops [1, 76]. Considering the requirement of freshwater and fertilizers, it is difficult to produce those fuels in a sustainable manners. The second generation biofuels are derived from cellulosic biomass feedstock using advanced biotechnological processes. Compared with the first generation biofuels, they hold greater promise because of their more favorable greenhouse gas emissions, no competition with food production, and less land usage [13, 60, 77-79]. However, the production of the second-generation biofuels faces several challenges: relatively high production cost, technological demands for enzyme, pretreatment and fermentation; and the essential development of a whole new infrastructure for harvesting, transporting and storing, and refining biomass [80]. Algae fuel are considered third generation biofuel [81]. Although algae fuels are superior to the other two types of biofuels in several aspects, developing a technological process for the production of the third generation biofuel is technologically more demanding than those of other types of biofuels. Here, we demonstrate that combining dilute acid hydrolysis with enzymatic hydrolysis can provide efficient saccharification of biomass of the invasive algae *Gracilaria salicornia*. Use of engineered *E. coli* KO11 successfully fermented monosaccharides from *G. salicornia* into ethanol (Fig. 4).

Pretreatment of feedstocks, especially those containing cellulose and hemicelluloses, and subsequent hydrolysis of these polysaccharides into monosaccharides are key steps in the production of second and third generation biofuels [25]. Dilute sulfuric acid method was applied for biomass hydrolysis as early as 1819 when
Braconnor retrieved fermentable sugar from linen [27]. Sulfuric acid was able to break the hydrogen bond among the cellulose polymers, making them available for sulfuric acid and further allowing it hydrolyzing glycosidic bonds to release sugars from cellulose and hemicelluloses [29, 82]. This dilute acid hydrolysis is still commonly used in today’s industrial processes [28]. However, dilute acid hydrolysis only yielded a low amount of monosaccharides from the invasive algal plants (Fig. 1 and 2). The enzymatic hydrolysis released much more monosaccharides from algal feedstocks than dilute acid hydrolysis alone (Fig. 3). To ferment monosaccharides derived from the invasive algae, genetically engineered \textit{E. coli} strain KO11, which is capable to ferment a variety of sugars (C$_6$ and C$_5$-sugars) [47, 54], was used in batch fermentation. After the complete consumption of glucose (detected by HPLC), the continuous production of ethanol from the invasive algae hydrolysate confirmed the metabolic ability of diverse sugars by this strain (Fig. 4 and S1). Furthermore, ethanol yield of this study was lower than those previously reported using extracts of the brown seaweed \textit{Laminaria hyperborean} [59] and the microalga \textit{Chlamydomonas reinhardtii} biomass [83]. The low ethanol yield may be ascribed to following reasons. First, macroalgal feedstocks have been reported to contain large amount of polysaccharides that are made up from monomers such as $\alpha$(1–3)-D-galactose-4-sulphate, $\beta$(1,4)-3,6-anhydro-D-galactose, $\beta$(1–3)-D-glucopyranose, D-mannitol, and mannose [55, 59]. Those sugars are not readily fermented by \textit{E. coli} KO11. Second, ethanologenic microbial strains used in the other studies are \textit{Zymobacter palmae} and \textit{Saccharomyces cerevisiae}, which have better capacities to ferment the previously mentioned sugars into ethanol than \textit{E. coli} KO11. Finally, hydrolysate of the invasive feedstocks may contain inhibitory chemicals of ethanol fermentation by \textit{E. coli} KO11.
Clearly, chemical analysis of biomass of the invasive species *G. salicornia* will be an interesting follow-up project. Metabolically engineered *E. coli* KO11 with capabilities of fermenting more monosaccharides derived from *G. salicornia* will surely increase the overall ethanol yield from invasive algal hydrolysate.

With increased demand for renewable biofuel, the development of technological processes for the production of second and third generation biofuels will be one of the greatest challenges facing our society. Production of third generation biofuel from invasive algal biomass will surely contribute to global efforts to reduce global warming and dependence on conventional fossil fuels. To that end, this study provides the first report on third generation biofuel production from marine environmental hazards.
Figure 1 Effect of sulfuric acid on the hydrolysis of the invasive algal feedstock.
Figure 2 Effect of temperature on the hydrolysis of the invasive algal feedstock.
Figure 3 Enzymatic hydrolysis of the invasive algal feedstock.
**Figure 4** Ethanol production from hydrolysate derived from invasive algal feedstock using the ethanologenic *E. coli* strain KO11.
Figure 5 Ethanol production from the glucose fermentation by the ethanologenic *E. coli* strain KO11.
Figure S1 HPLC analysis of sugars and ethanol in media at different fermentation times.
Table 1 Measurement of glucose concentration released from algal feedstock by various methods and the potential for ethanol fermentation.

<table>
<thead>
<tr>
<th>Method</th>
<th>Glucose Release (mg/g wet algal biomass)</th>
<th>Maximum Ethanol Production (mg/g dry algal biomass)</th>
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<td>Physical and Chemical Treatments</td>
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<td>Enzymatic hydrolysis</td>
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Chapter 3. The microbial community structures associated with two pairs of competitive Hawaiian native and invasive macroalgae

Abstract

Marine macroalgae are known to harbor large populations of microbial symbionts, and yet, microbe symbiosis in invasive macroalgae remains largely unknown. In this study, we applied molecular methods to study microbial communities associated with two invasive algae Acanthophora spicifera and Gracilaria salicornia and the two native algae G. coronopifolia and Laurencia nidifica at spatial and temporal scales in Hawaiian coral reef ecosystems. Bacterial communities of both the invasive and native macroalgae displayed little spatial or temporal variation, suggesting consistent and stable bacterial associations with these macroalgae. Results of this study identified three types of bacterial populations: non-specific (present in both algal and water samples); algae-specific (found in all algal species but not water samples); and species-specific (only found in individual species). The bacterial diversity of invasive algae was lower than that of their native counterparts at phylum and species levels. Notably, the vast majority (71%) of bacteria from communities associated with the invasive algae G. salicornia were representatives of Cyanobacteria, suggesting a potential ecological significance of symbiotic cyanobacteria. Collectively, the algae associated microbial community profile provided a guide for potential microbes that can be screened for application in biofuel production.
Introduction

How biotic and abiotic environmental factors control the abundance and diversity of species and consequently influence the ecosystem function and stability has become a major topic in ecology [84-86]. Biological invasions, recently enhanced by trade globalization and climate warming, have become one of the most serious threats to local biodiversity and ecosystem function in both marine and terrestrial ecosystems [85, 87]. Investigation of biological invasion has largely focused on its economic and environmental impact and management of exotic species [85, 88]. Although microbial symbionts are ubiquitous with exotic species and known to have profound influences on the evolution and function of their hosts [89-94], investigation of microbial communities associated with alien species, particularly invasive ones, is rare [87].

Marine macroalgae are a key component of global biosphere with a critical role in the stability, function, and health of marine ecosystems [61, 62]. They yield high primary production, playing a crucial role in the global carbon cycle and the reduction of global warming [62]. Algae can also cause environmental problems when introduced to a new ecosystem because of the risk of becoming an invasive species in their newly introduced environment. As a matter of fact, invasive algae have frequently been reported to alter ecosystem structure, to reduce indigenous biodiversity, and to cause economic losses [23, 64]. There are over two dozen alien algal species in Hawaii and about half of them have become invasive [22]. Acanthophora spicifera and Gracilaria salicornia are two commonly found invasive algal species in Hawaiian coral reef ecosystems [65]. Acanthophora spicifera successfully competes for space with the native algae Laurencia
*nidifica* and *G. salicornia* competes with native *G. coronopifolia* and other reef algal species [65].

Microbial communities have long been found to exist in marine macroalgae but their ecology and interactions with the hosts are poorly understood. A limited numbers of studies have focused on epiphytic bacterial communities with a focus on the culturable communities [95-98]. Epiphytic bacteria are thought to play a key role in the colonization process of an algal thallus because they may serve as a nutrient source and provide growth factors for their algal hosts [95, 96, 99-102]. Endosymbiotic bacteria are also reported to be commonly present in marine macroalgae [103-105]. Due to their abundance in marine macroalgae, microbial communities may have significant ecological implications on the biology of invasive species. In this study, we applied various molecular approaches (e.g. DGGE and 16S rRNA gene library construction) to investigate the diversity of bacterial communities associated with 4 Hawaiian marine algal species (2 invasive and 2 native). We hypothesize that invasive marine algae harbor a microbial community structure that is different from the native one. Thus, the ecological study may unravel important microbial groups associated with invasive algae and provide insight for further functional study of these microbes on the biology of invasive species.

**Materials and methods**

*Sample collection and DNA extraction*

Algal thalli of *Acanthophora spicifera*, *Gracilaria salicornia*, *G. coronopifolia*, and *Laurencia nidifica* were collected at two sites (S1 and S2). Site 1 is located on reef in subtidal zone near Waikiki. Site 2 is on reef in shallow intertidal zone of Kaimana Beach.
All specimen were collected in their healthy conditions. For the temporal analysis, samples of these species were collected from these two sites in September 2007 (T1) and July 2008 (T2). *Gracilaria salicornia* is mostly found in intertidal and shallow subtidal zones to a depth of 4 m and its thallus is usually 18 cm tall forming low tangled mats to 30 cm broad. *Gracilaria coronopifolia* has erect and bushy thallus, forming irregular dichotomous branching up to 15 cm tall, but mostly 6-8 cm and commonly found in the shallow subtidal zones to the depth of 4 m. Members of the genus *Gracilaria* are characterized by protuberant cystocarps and microscopically, a medulla of large rounded cells grading to a small-celled cortex. The thalli of *A. spicifera* are erect and usually grows up to 40 cm tall and commonly found in reef flats, tide pools and rocky intertidal beaches. It has the upright axes that are cylindrical with short spirally arranged and spine like branches with clusters of white or colorless hairs. Finally, the thallus of *Laurencia nidifica* is erect, usually 10 cm tall and has uniaxial terete axes with terminal pits. Its thallus has four pericentral cells per axial cell, commonly found in reef flats and lower intertidal zones to 3 m deep.

For each species, 3 entire individual thalli were sampled for library construction and DGGE analysis [106]. Seawater samples were collected at each sample collection site as a control. Both algal and seawater samples were transported in a cooler immediately after collecting to laboratory for processing. Algal thalli were rinsed 3 times with sterilized artificial seawater to remove loosely attached bacteria, dried briefly with paper towels and immediately used for DNA extraction. Seawater samples were sequentially filtered through 2 and 0.2 μm cellulose-acetate filters. The filters were frozen in liquid nitrogen until further processing. Total genomic DNA of algae and
filters of seawater samples were extracted using the FastDNA kit (MP Biomedicals) following the manufacturer’s instruction. The resulting DNA was purified using QIAquick PCR Purification Kit (Qiagen) and used as PCR templates.

**Molecular fingerprinting analysis of microbial communities**

Bacterial 16S rRNA gene fragments for denaturing gradient gel electrophoresis (DGGE) were amplified using primers of 341GC, 907rC and 907ra [107]. The PCR reaction consists of 1x reaction buffer, 1.5 mM MgCl₂, 200 μM dNTPs, 0.4 μM forward primer of 341GC, 0.2 μM reverse primer of 907rC, 0.2 μM reverse primer of 907ra, approximate 20 ng of bacterial DNA, and 1.25 unit of Taq DNA polymerase in a final volume of 50 μL. The 16S rRNA gene fragments were amplified using a touch-down PCR procedure as follows: initial denaturation at 94°C for 3 min, followed by 20 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s with every cycle decreasing by 0.5°C, and elongation at 72°C for 40 s; thereafter another 30 cycles of denaturation at 94°C for 40 s, annealing at 50°C for 30 s, elongation at 72°C for 40 s, and finally elongated at 72°C for 10 min. DGGE analysis was performed using a model DGGE-2001 electrophoresis system (C.B.S Scientific Company Inc., CA.) with a denaturing gradient of 30-70% in a 10% polyacrylamide gel, with bands of interest cloned for sequencing analysis following methods described previously [108]. Up to 4 colonies from each mini-library constructed from the 10 excised DGGE bands were picked and sent for sequencing.

The 16S rRNA gene fragments for Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis were amplified using a Fam-labeled forward primer of
27f and universal reverse primer of 1492r. The PCR products were purified and enzyme digested by *Hae*III (Promega, Madison, WI) before fragment analysis.

*Construction and analysis of 16S rRNA gene library*

The total genomic DNA was used as PCR templates for the amplification of 16S rRNA genes using the primers 341f and 1406r [109]. Construction of 16S rRNA gene library was performed using method described previously [110]. Positive clones were grouped based on restriction fragment length polymorphisms (RFLP) patterns using the restriction enzyme *Hae*III (Promega, Madison, WI). Two representative clones from each group were cultured for plasmid DNA isolation and subsequent sequencing analysis.

*Sequence and phylogenetic analysis*

Inserts of plasmids were sequenced at the University of Hawaii DNA Core Sequencing Facility on an Applied Biosystems 3730XL automated DNA sequencer. Sequence data were edited with Chromas Lite, version 2 (Technelysium). Chimeric sequences were checked using the Ribosomal Database Project (RDP) II Chimera Check program (http://rdp8.cme.msu.edu/cgis/chimera.cgi). Clone sequences with 97% or above identity were grouped as an operational taxonomic unit (OTU) or one “species” based on average neighbor method using DOTUR [111]. One sequence from each OTU was selected as a representative for further analysis. For preliminary identification, sequences of bacterial 16S rRNA genes were compared with those deposited in the NCBI database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov). Sequences of 16S rRNA genes were additionally compared with those in RDP using Classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp). The representatives of bacterial 16S rRNA sequences in this study and the matched sequences from GenBank
were aligned using BioEdit, version 7.0.5.3 [112]. Phylogenetic trees were constructed using the neighbor-joining method implemented in PAUP* 4.0b10 described by Gao et al [108]. The quality of the branching patterns for NJ trees was assessed by bootstrap analysis after randomly resampling 1,000 replicates. The percentage coverage \( C \) of the clone libraries was calculated according to the following equation: \( C = 1 - (n_i/N) \times 100 \) [113, 114], where \( n_i \) is the number of unique clones, and \( N \) is the total number of clones in the library. The diversity within the libraries was measured with the Shannon-Weaver index \( (H) \) and evenness \( (E) \) using the equation \( H = -\sum P_i \ln(P_i) \), where \( P_i \) is the proportion of the total number of OTUs made up to the \( i \)th OTUs, and \( E = H/\log(S) \), where \( S \) is the total number of OTUs in the community.

**Nucleotide sequence accession number**

Bacterial 16S rRNA gene sequences obtained in this study were deposited in GenBank under accession numbers HM474862-HM475010.

**Results**

**Molecular fingerprinting analysis of microbial communities**

To reveal the features of microbial communities associated with native and invasive algae, three individual thalli of two invasive algal species, *i.e.*, *Gracilaria salicornia* and *Acanthophora spicifera*, and two native species, *i.e.*, *G. coronopifolia* and *Laurencia nidifica*, were collected from the two sampling sites. DGGE analysis indicated that microbial band patterns derived from algal thalli varied among different algal species. However, band patterns derived from different individual thalli of the same species displayed little variations, suggesting similar microbial communities in algal thalli of the same species (Fig. 1a-c). Additionally, band patterns derived from algal thalli of the same
species displayed little variations among the two collecting sites. The presence of similar microbial communities in this example indicated the little variation of algal species-specific microbes in a closely sampled ecosystem. However, spatial stability of microbial community associated with the same algae would require further evidence supporting the similar microbial community associated with distantly sampled algae species. Lastly, band patterns of microbial communities associated with both the invasive algae G. salicornia and the native algae G. coronopifolia collected at two different times were each also similar (Fig. 1c). Hence, microbial communities associated with the invasive and native algal species showed little temporal variations.

DGGE analysis revealed three groups of bacteria in four algal species (Fig. 1a-c). The first group of bacteria was nonspecific and present in both seawater and four algal species (Bands 1a & 2a in Fig. 1a and band 1 in Fig. 1c). The second group was those microbes found only in algae (Band 3a in Fig. 1a and band 2 in Fig. 1c) and the third one was species-specific group, present either in native or invasive algal species (Bands 4a, 5 & 6 in Fig. 1a and bands 3 & 4 in Fig. 1c). The greatest diversity of microbial communities was found in the native species L. nidifica and the second greatest in the invasive species A. spicifera. The microbial diversity in the native species G. coronopifolia was comparable to that in the invasive species G. salicornia. Results of T-RFLP analysis revealed more distinct diversity pattern between invasive and native algae, i.e. the native algae harbored greater diversity of bacteria compared to invasive algae (Fig. S1). Overall, our results suggested that invasive and native species harbored different bacterial lineages, but also contain stable and species-specific microbial phylotypes.

Phylogenetic affiliation of phylotypes derived from DGGE bands
To further identify three types of microbes mentioned previously, DGGE bands as indicated in figure 1a and 1c were excised and used to construct clone libraries. Besides plastid sequences, sequencing analysis identified 4 bacterial phyla: Actinobacteria, Cyanobacteria, Deinococcus-Thermus, and Proteobacteria (Alpha- and Gamma- and Deltaproteobacteria) (Fig. 2). Plastid sequence of marine algae was found in 4 DGGE bands (1a & 3a in Fig. 1a; 1 & 3 in Fig. 1c). Two plastid OTUs (GsGc1-1 & GsGc3-1) derived from G. salicornia and G. coronopifolia were closely (96%) related to plastid sequence of G. tenuistipitata. Three plastid OTUs (AsLn1-4, AsLn3-2, & AsLn3-3) derived from L. nidifica and A. spicifera were closely related (97%, 98%, and 99%, respectively) to uncultured cyanobacterial sequence TAU-7-68 from marine sponges and distantly (93%) to the plastid sequence of Neoptilota densa (Fig. 2).

Phylogenetic analysis of sequences derived from DGGE bands revealed 4 Marine Bacterial Symbiont (MBS) groups (Fig. 2). Members of MBS 1 included 3 cyanobacterial OTUs (AsLn2-4, AsLn3-1, & AsLn5-4), which were closely affiliated with coral associated cyanobacterial sequences. MBS 2 included 4 alphaproteobacterial OTUs (GsGc2-2, AsLn4-1, AsLn6-4, & AsLn6-5) that were affiliated with bacterial symbiont sequences derived from marine sponges, abalone and corals. MBS3 only contained 1 deltaproteobacterial OTU (AsLn4-2) that was related to bacterial clone sequences from other marine animals. Furthermore, 1 actinobacterial OTU (AsLn4-3) belonged to MSB 4 shown close (99%) affiliation with actinobacterial phylotype UA21 from the marine algae Ulva australis [106]. Interestingly, this actinobacterial phylotype is also present in the marine algae Delisea pulchra in the same study. Finally, the rest of OTUs identified
from DGGE band clone libraries were found to be related to bacterial 16S rRNA gene sequences from marine sediments, coral reef, microbial mat, and marine plant (Fig. 2)

Diversity and phylogenetic analysis of 16S rRNA gene clone libraries

PCR-based 16S rRNA gene clone libraries were constructed for further comparison of bacterial communities within these 4 algal species using samples collected in November 2007. Sequence analysis identified 9 bacterial phyla: Proteobacteria (Alpha-, Delta-, Gamma-, and unclassified), Acidobacteria, Chloroflexi, Verrucomicrobia, Cyanobacteria, Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes, and unclassified bacteria. Additionally, plastid sequences were recovered from clone libraries derived from A. spicifera (n=24), G. salicornia (n=6), and G. coronopifolia (n=3). Diversity indices of these clone libraries are summarized in Table 1. Finally, members of Euryarchaeota were only detected in the libraries constructed from the native alga G. coronopifolia (n=1) and seawater (n=1).

Analysis of 16S rRNA gene sequences indicated a great variability in composition of bacterial phylotypes among 4 algal clone libraries (Fig. 3). At species level, 49 OTUs were identified in the library of the native alga L. nidifica, while only 16 bacterial OTUs were found in the library of the invasive alga A. spicifera. 24 bacterial species were recovered from the library of the native alga G. coronopifolia and 22 bacterial species from the library of the invasive alga G. salicornia. The highest phylum-level diversity was found in the library of the native alga L. nidifica and the lowest in the invasive G. salicornia.

Combined with DGGE libraries, there were total of 127 OTUs (111 OTUs from clone library and 16 OTUs from DGGE library) obtained from bacterial 16S rRNA gene
sequences derived from the 4 algal species. Most of these OTUs were associated with single algal species and 30 OTUs with multiple species. Of these OTUs associated with single species, 9 and 10 were unique to the invasive algal species *A. spicifera* and *G. salicornia*, respectively, while 33 and 8 to the native algal species *L. nidifica* and *G. coronopifolia*, respectively. There were also 4 OTUs unique to DGGE library from the algae pair of *A. spicifera* and *L. nidifica*. The last 2 OTUs were found to be clustered with seawater sequences. 2 alphaproteobacterial phylotypes (GC03, Ln08 and AS36; GC24, Gs40 and Ln13) were presented in 3 algal species and 1 actinobacterial phylotype (Gc55, Gs2-5, Ln01, and AS02) in all 4 algal species (Fig. S2). The bacterial diversity derived from clone libraries was consistent with that of T-RFLP analysis with the greatest diversity found in the native species *L. nidifica* and the least in the invasive species *G. salicornia*.

Additionally, the clone library derived from the invasive algae *G. salicornia* was largely dominated with cyanobacterial sequences (71%, n=68) and proteobacterial sequences (19%, n=18), while the clone library derived from the native algae *G. coronopifolia* only contained 28% (n=18) of cyanobacterial sequences with proteobacterial sequences as the most abundant phylotypes (39%, n=25) (Fig. 3). Bacterial phylotypes of the clone library derived from the invasive species *A. spicifera* were dominated with algal plastid sequences (40%, n=24) and proteobacterial sequences (23%, n=14). On the other hand, the native species *L. nidifica* contained predominately actionobacterial (29%, n=30) and proteobacterial (28%, n=29) sequences. Although alphaproteobacterial sequences were recovered from all 4 clone libraries, the two native algal species (23% for *L. nidifica* and 25% for *G. coronopifolia*) contained higher
abundance of proteobacterial phylotypes than that of the two invasive algal species (17% for *A. spicifera* and 18% for *G. salicornia*) (Fig. 3). Overall, the bacterial diversity of the native algae was greater than that of invasive species at both phylum-level and species-level (Fig. 3 and Table 1).

**Discussion**

Marine macroalgae are known to contain large populations of bacteria with potentially important impact on the physiology and ecology of their algal hosts [96, 115]. Although some efforts have been made to study epibacterial communities on macroalgae [96, 116], the evidence on macroalgae associated bacterial communities is still scarce. This study represents the first report that compares the diversity of microbial community associated with invasive and native marine algal species in the same ecosystem at a temporal scale.

Marine eukaryotes, which along history establish distinct bacterial populations different from those in the water columns, have been shown to be a substantial reservoir of new bacterial diversity [106, 117]. Bacterial communities associated with these marine eukaryotes have been examined for several marine organisms, including but not limited to, corals [118-122], tubeworms and bivalves at hydrothermal vents [123, 124] and sponges [117, 125, 126]. Although there are exceptions, it seems to be generally agreed that host-specific microbes exist in marine eukaryotes with variations at taxonomic levels of these hosts. Particularly, sponges, the best-characterized marine eukaryotes in this regard, have been reported to harbor the stable and sponge-specific microbial phylotypes among many sponge species across different ocean provinces [125, 127, 128]. Information on the host-specificity of bacterial community associated with macroalgae is
somewhat limited because few studies have been carried out to analyze the whole community of bacteria in marine algae. Bacteria on macroalgae surfaces can typically reach the densities of approximately $10^7$ bacterial cells per cm$^2$, with diverse ecological functions and implications [96, 116, 129]. Bacterial communities associated with the kelp *Laminaria saccharina* fluctuated with different parts of the alga and also displayed seasonal and geographical difference [98]. Analysis of bacterial community associated with the red alga *Delisea pulchra* and the green alga *Ulva australis* from Bare Island and Shark Point, respectively, (Sydney, Australia) revealed different phylum-level diversity (7 vs 4) and species richness (79 vs 36) among two species. Diverse proteobacterial (*Alpha*-, *Beta*-, *Delta*-, and *Gamma*-) phylotypes, as well as members of the *Bacteroidetes*, *Plantomycetes* and *Cyanobacteria* were identified in the bacterial communities associated with the invasive green alga *Caulerpa taxifolia*, with the *Alphaproteobacteria* as the predominant phylotypes found in all samples collected from Australia, Tahiti, the Philippines, and the Mediterranean [115]. The major bacterial lineages associated with this invasive algal specie differed significantly among biogeographical locations. This study revealed different phylum-diversity and species richness among native and invasive algal species (Fig. 3). The diversity of invasive macroalgae is generally lower than that of their native counterparts, which is more like the case (16 vs. 49) in the exclusively competitive pair of algae *A. spicifera* and *L. nidifica* (Table 1).

This study identified several bacterial phylotypes shared by multiple algal species. One actinobacterial phylotype (AS02, AS58, Ln01, Gs2-5 and Gc55) was found in all invasive and native Hawaiian species samples in this study (Fig. S2d). This finding is a
contrast to other studies. For example, analysis of bacterial community associated with the red alga *D. pulchra* and the green alga *U. australis* indicated that there was no overlap between bacterial communities on these algal species [106]. Our DGGE analysis also revealed the shared DGGE bands among the invasive and native algal species (Fig. 1a-c). Additionally, most of the alphaproteobacterial sequences derived from the invasive *C. taxifolia* were affiliated to the *Rhodobacter* group [115]. This bacterial group has commonly been found in marine algae [106, 130] and has been suggested to play a role in gall (tumor) formation on macroalgae of the genus *Prionitis* [130]. Phylogenetic analysis of this study indicated the shared OTUs were closely affiliated with bacterial sequences from marine macroalgae and other marine eukaryotes. As revealed in the study of bacterial sequences from the algae *D. pulchra* and *U. australis* [106], sequences derived from the Hawaiian algae were clustered together, but interspersed by sequences from sponges or corals. Because of the paucity of alga-derived bacterial sequences, it is apparently premature to reach any conclusion on the existence of algal-specific monophyletic sequence clusters.

Cyanobacteria are the most predominant bacterial phylotypes associated with the invasive species *G. salicornia*, accounting for 71% of total bacterial sequences (Fig. 3). Nitrogen-fixation by cyanobacteria associated with macroalgae have been described for many species, including pelagic and benthic species of *Sargassum* [131], a variety of coral reef species [132], and *Enteromorpha* spp. on temperate sand flats [133]. They have commonly been postulated to benefit their algal hosts by transfer of bacterially fixed nitrogen to the host plant. Indeed, nitrogen-fixation has been estimated to provide anywhere from 2 to 140% of the nitrogen requirement of various macroalgae-microbe
associations [131, 132, 134]. Furthermore, a particularly high efficiency of nitrogen-fixation product transfer has been predicted for endophytic cyanobacteria [134]. Transfer of fixed-nitrogen from symbiotic cyanobacteria to their hosts also occur in various macrophyte hosts, including lichens, bryophytes, aquatic ferns, and the angiosperm *Gunnera* spp.. In these associations, up to 90% of the fixed-nitrogen, including other photosynthetic products, is excluded by the symbiotic cyanobacteria and taken up by the host [135-139]. The large populations of cyanobacteria associated with *G. salicornia* may contribute to the growth of their hosts and provide advantage in outcompeting over its native counterpart. Therefore, investigation of true contribution of cyanobacterial populations to the invasive nature of *G. salicornia* is in progress. The population of cyanobacteria in the invasive *A. spicifera* was not as dominant as that in *G. salicornia* (Fig. 3). However, compared to its competitive native *L. nidifica*, the invasive *A. spicifera* was found to be predominant (40%) with algae plastid (Fig. 3) that is believed to evolutionally originate within the cyanobacterial divergence [140]. These data collectively suggests an important role played by algae plastid or cyanobacteria on the biology of invasive algae.

Alphaproteobacteria are the most commonly found bacterial phylotypes in the invasive algae *C. taxifolia* [115]. In this study, alphaproteobacteria were found to be abundant not only in invasive species *G. salicornia* and *A. spicifera* but also in their competitive native counterparts *G. coronopifolia* and *L. nidifica* (Fig. 3). Nevertheless, results of this study are consistent with the previous reports that alphaproteobacteria are typical bacterial phylotypes associated with marine macroalgae [98, 106, 115, 116]. This group of bacteria is of particular interest to studies of marine microbial symbionts
because some members of this group are identified in many marine sponges or other marine eukaryotes, others able to reduce or oxidize nitrogen, and still some others contribute significantly to dimethylsulfoniopropionate (DMSP) in the oceans [117, 141, 142]. The close affiliation of the majority of alphaproteobacterial OTUs resulted from this study with marine bacterial symbionts clearly indicated their ecological significance in the Hawaiian algae symbiosis. Notably, many alphaproteobacterial OTUs are novel bacterial phylotypes that are not readily clustered with their phylogenetic neighbors in GenBank (Fig. S2).

Finally, one interesting observation of this study is that invasive species generally contain less diverse bacteria than their native counterparts (Fig. 3 and Fig. S1). This is similar to the finding that the abundance of epibiota were lower in the invader Fucus evanescens than that of native F. vesiculosus [61]. Also, results of this study seem to be consistent with the reports that large proportions of invasive terrestrial plants have low mycorrhizal communities [85]. Unfortunately, the nature of association between plant-microbe symbioses in both terrestrial and marine environments remains largely unknown. Given the abundance of microbes associated with macroalgae and the significance of microbial symbionts in the invasion of plants and insects [85, 143], species-specific microbial symbionts may have profound implications for biological invasion of macroalgae. Our understanding of the ecology of marine algal invasion is still limited and most studies refer to the known mechanism related to terrestrial invasion ecology (for review, refer to [61]). Molecular biological techniques have always been considered as a powerful tool for ecological study [144] and have been actually developed and applied in the study of algal invasion [145-147]. Our understanding of macroalgae-microbe
symbioses at molecular level is rather poor. This study represents the first step to unravel the microbial symbioses of invasive algae, which is not well understood but vitally important for our understanding of algal invasion.
Figure 1 DGGE analysis of microbial community associated with the invasive algae *Acanthophora spicifera* (As) and *Gracilaria salicornia* (Gs), the native algae *Laurencia nidifica* (Ln) and *G. coronopifolia* (Gc), and seawater (SW) samples. a and b. DGGE comparison of microbial communities associated with the algal pairs for As/Ln and Gs/Gc, respectively, from two different sampling sites. c. DGGE comparison of microbial communities associated with the algal pair of Gs/Gc collected from two different times. Cloned DGGE bands are labeled with dot and number.
Figure 2 Neighbour-joining tree based on 16S rRNA gene sequences derived from DGGE mini clone libraries. Sequences obtained in this study were indicated by library name followed by band number and colony number, e.g., GsGc1-1 means that the sequence was obtained from colony 1 in the mini clone library constructed using the excised band 1 of Gs/Gc comparison in the DGGE gel.
**Figure 3** Frequencies of bacterial phylotypes in five 16S rRNA gene libraries derived from bacterial community associated with the invasive algae *Acanthophora spicifera* (*As*) and *Gracilaria salicornia* (*Gs*), the native algae *Laurencia nidifica* (*Ln*) and *G. coronopifolia* (*Gc*), and seawater (SW).
Figure S1 T-RFLP analysis of bacteria associated with Hawaiian invasive algae *Acanthophora spicifera* (*As*) and *Gracilaria salicornia* (*Gs*), native algae *Laurencia nidifica* (*Ln*) and *G. coronopifolia* (*Gc*), and seawater samples (SW).
Figure S2 Neighbor-joining phylogenetic trees (Cyanobacteria/Algae Plastid (a), Alphaproteobacteria (b), Beta-, Gamma- and unclassified-Proteobacteria (c), and the other bacterial groups (d), based on 16S rRNA genes sequences of bacteria from marine algae and seawater. Numbers above or below branches indicate bootstrap values (≥50%) from 1,000 replicates. Sequences from this study were indicated in bold with the name abbreviation of algal species followed by the colony ID. See the legend in Fig. 1 for the abbreviation of algal species.
Table 1 Diversity indices of five 16S rRNA gene clone libraries derived from microbial community associated with algae and seawater samples. ‘As’, ‘Ln’, ‘Gs’, ‘Gc’ and ‘SW’ represent libraries derived from microbial community associated with algae *Acanthophora spicifera, Laurencia nidifica, Gracilaria salicornia, G. coronopifolia* and seawater samples, respectively.

<table>
<thead>
<tr>
<th>Library</th>
<th>No. of Clones (OTU)</th>
<th>Coverage(^a) (%)</th>
<th>Shannon-Weaver Index ((H^b))</th>
<th>Species Richness ((S^d))</th>
<th>Evenness ((E^c))</th>
</tr>
</thead>
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<tr>
<td>As</td>
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<td>16</td>
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<tr>
<td>Ln</td>
<td>103 (49)</td>
<td>79</td>
<td>3.70</td>
<td>49</td>
<td>0.95</td>
</tr>
<tr>
<td>Gs</td>
<td>96 (22)</td>
<td>88</td>
<td>2.05</td>
<td>22</td>
<td>0.66</td>
</tr>
<tr>
<td>Gc</td>
<td>65 (24)</td>
<td>83</td>
<td>2.90</td>
<td>24</td>
<td>0.91</td>
</tr>
<tr>
<td>SW</td>
<td>64 (24)</td>
<td>80</td>
<td>2.73</td>
<td>24</td>
<td>0.86</td>
</tr>
</tbody>
</table>

\(^a\)Coverage = 1-(\(n1/N\)), where \(n1\) is the number of OTU’s appearing only once in the library and \(N\) is the total number of clones examined.

\(^b\)\(H = -\sum P_i \ln(P_i)\)

\(^c\)\(E = H/\ln(S)\)

\(^d\)\(S = \) total number of OTUs in the community
Chapter 4. Bioprospecting of cellulolytic bacteria for consolidated bioprocessing

Abstract

Consolidated bioprocessing (CBP)—a biological process using a single microbe to perform saccharification and fermentation on lignocellulosic biomass to produce desired end products—is considered a valuable route for next generation biofuel production. Two strategies, known as native and recombinant strategy, are generally available for developing candidate bacteria involved in this process. However, due to the lack of efficient candidate bacteria, the development of CBP is limited. In this study, three cellulolytic facultative anaerobes have been isolated from various environmental sources. A cellulolytic bacterium FNP1 was isolated from human feces and identified as an *E. coli* strain that could be a valuable candidate for easy genetic manipulation using native CBP microbial development strategy. Multiple aerobic cellulolytic microbes were also isolated, one of which TF2 holds great value being the candidate for heterologous cellulase expression involved in the recombinant CBP microbial development strategy.
**Introduction**

Biofuel is considered a viable alternative to fossil fuels for the sustainable development of our societies. In the face of energy crisis and serious environment concerns including atmospheric pollution, greenhouse gas emission, etc. [60], biofuels fulfill our need for energy sources that have features of net energy gain, having environmental benefits, economically competitive, and can be produced in large quantities without reducing food supplies [1].

Major platforms for biofuel production include fuels derived from microalgae and lignocellulosic biomass. Microalgae have high photosynthetic efficiency and can store large amounts of carbohydrate and oil inside their cells [148]. The large quantity of lipid can be transformed into biodiesel through transesterification [149], and the carbohydrate biomass can be saccharified into sugar stock for the fermentation of ethanol and other types of biofuels [150]. Other routes of microalgae based biofuel production include syngas production from algal biomass [151], direct hydrogen production [152], among others. Although this approach could potentially revolutionize the future of biofuel production, the higher overall production cost compared to that of fossil fuels still hinders its industrial application. Several concerns need to be addressed before further development, which includes maximizing the oil production, increasing cell growth rate, lowering the cost of growing and harvesting algae [148], and the identification of key chemical inducers involved in metabolic change [11]. On the other hand, lignocellulosic biomass derived biofuel production via microbial conversion could be proposed as a platform for the fully renewable production of transportation fuels [2]. Currently in the states, corn (*Zea mays*) grain and other cereals like sorghum (*Sorghum bicolor*) constitute
the primary feedstock for ethanol production. However, these feedstock are not sustainable for long term application because of the competition for land use in food supplies [18]. Abundant plant biomass and other lignocellulosic and cellulosic waste serve as an economic alternative, provided several key challenges are readily overcome [2, 13]. Before biomass can be used for fermentation, it needs to be pretreated to release long-chain polysaccharides, especially cellulose and hemicelluloses, and requires subsequent hydrolysis to convert these polysaccharides into their component sugars [25]. One of the challenges that potentially increases the biofuel production cost in the two stage platform is the need for large quantities of glycoside hydrolases in order to release component sugars from these rigid lignocellulose [2]. Consolidated bioprocessing (CBP), which combines saccharification and fermentation into a single step, uses a single microbe to perform sugar hydrolysis and biofuel production, and it could potentially minimize the costs dedicated in the enzyme generation step [2, 35]. The microbe–enzyme synergy also proved to have higher hydrolysis rate up to 4.7 fold compared to the purified enzyme from the same microbe [153].

Two strategies are currently available for developing suitable microbes to facilitate CBP, namely the native and recombinant cellulolytic strategies [35]. The recombinant strategy engineers a heterologous cellulase system into non-cellulolytic microbes that feature the production of desired end products to facilitate the hydrolysis and fermentation of pretreated lignocellulose biomass. Various microbes, including the yeast *Saccharomyces cerevisiae* [154], and bacteria *Klebsiella oxytoca*, *Zymomonas mobilis* and certain *E. coli* strains, have been widely reported for this purpose [35]. A recent demonstration of an *E. coli* strain represent a major advance towards a realized
CBP, in which *E. coli* was introduced with various cellulotic enzymes and directed to produce three advanced biofuel suitable for gasoline, diesel and jet engines [2]. Conversely, the native strategy engineers a natural cellulolytic microbe to produce the desired end product and titer. *Clostridium cellulolyticum* [155] and *C. thermocellum* [156] represent the most utilized microbes for this purpose. The lack of suitable candidate microbes has impeded the development of the native cellulolytic strategy. The lignocellulose are naturally degraded by microbial communities [157, 158], which could potentially overcome the challenge of expressing the high amount of heterologous cellulase required in the recombinant strategy. Thus, it is imperative to discover more natural cellulolytic microbes in order to facilitate the development of CBP.

In this study, various sources for isolating both aerobic and anaerobic cellulolytic bacteria were explored, and preliminary studies on the obtained bacteria showed their great potential in further CBP development.

**Materials and methods**

*Collection of isolation sources*

Samples of soil and marine sediment were collected in various places on the island of Oahu. The specimen was sealed in sample bags and mixed with enrichment media as soon as they were returned to the lab. Termites were collected in rotten woods and were meshed to expose their guts before incubation in the enrichment media. Human feces samples were collected fresh and directly injected into the enrichment media.

*Isolation of cellulose-degrading bacteria*

The enrichment media containing the following percentage components (0.6 NaCl, 0.1 (NH₄)₂SO₄, 0.05 KH₂PO₄, 0.05 K₂HPO₄, 0.01 MgSO₄, 0.01 CaCl, 0.5 cellulose
microcrystalline (or carboxymethyl cellulose (CMC) or cellulose filter paper) and 0.05 resazurin) were prepared for enriching the cellulose degrading microbes in different isolation sources. Enrichment media containing samples derived from soil, sediments, or termite guts, were incubated aerobically at 30 °C for 7 days. The enrichment media inoculated with human feces samples were incubated at 37 °C for the same period of time. A separate, anaerobic set of enrichment cultures was prepared in 50 ml vials by flushing with nitrogen for 5 min, and were then incubated in anaerobic conditions.

The cellulose Congo-Red agar medium (0.5 g/L KH\textsubscript{2}PO\textsubscript{4}, 0.25 g/L MgSO\textsubscript{4}, 2 g/L cellulose, 0.2 g/L of Congo-Red, 2 g/L of gelatin and 15 g/L of agar; pH 6.8-7.2) was used to isolate cellulose-degrading bacteria from the enrichment cultures. As cellulolytic bacteria will form a clear zone around the growth on the Congo-Red cellulose agar, it provided a rapid and sensitive test to indicate if certain isolates were hydrolyzing cellulose. Colonies with a positive reaction were cultured for further investigation.

**Identification of cellulolytic bacteria**

Cellulolytic bacteria were identified by sequencing their 16S rRNA genes and performing a BLAST search in the NCBI database. Colony PCR was performed on each isolate in a 50 µl PCR reaction containing 1× PCR buffer (Promega), 1.5 mM Mg\textsuperscript{2+}, 200 µM dNTP, 0.2 µM 27F primer (5'-AGAGTTTGATCCTGGCTCAG-3'), 0.2 µM 1492R primer (5'-GGTTACCTTGGTACGACTT-3') and 5 u Taq polymerase. The PCR products were then purified using the Promega Wizard® Genomic DNA Purification Kit and subjected to sequencing analysis.

**Qualitative measurement of cellulase activity for aerobic cellulolytic isolates**
Aerobic cellulolytic isolates were inoculated into basal salt media ([161] [(g/L)] NaNO₃ 4.0, NaCl 1.0, KCl 1.0, CaCl₂·2H₂O 0.1, KH₂PO₄ 3.0, Na₂HPO₄·12H₂O 3.0, MgSO₄ 0.2, FeSO₄·7H₂O 0.001 plus 500× trace element stock solution consisting (g/L): FeCl₃·6H₂O 0.08, ZnSO₄·7H₂O 0.75, CoCl₂·6H₂O 0.08, CuSO₄·5H₂O 0.075, MnSO₄·H₂O 0.75, H₃BO₃ 0.15, Na₂MoO₄·2H₂O 0.05; pH 6.8.), supplemented with cellulose filter paper (0.1% w/v) as the sole carbon source for measurement of total cellulase ability. Control media was prepared without bacteria inoculation and all culture media were incubated at 37 °C at 180 rpm for up to 3 days. After incubation, glucose concentration in the media was measured by refractive index detector using HPLC [162].

Carboxymethyl cellulose (CMC) fermentation by facultative cellulolytic anaerobes

MOPS minimal media with supplements [40× ACGU Solution and 20× Supplement EZ (TEKnova)] was used as fermentation media. 25 ml of fermentation media was prepared in the autoclaved fermentation vials from 10× MOPS mixture, 100× K₂HPO₄ (0.132 M), 40× ACGU Solution, 20× Supplement EZ, 10× CMC (1%) solution and trace resazurin. 1 ml of fermentation media was withdrawn from the fermentation system before the system was set up. In the meantime, a facultative anaerobic isolate was inoculated into 5 ml of nutrient broth until OD₆₀₀ reached 1.0. A bacteria pellet was collected by centrifuging at 5,000 g for 10 minutes, and washed twice with distilled water. The bacteria was resuspended in 1 ml of fermentation media and injected into the fermentation system. The fermentation system was then sealed airtight and flushed with N₂ for 5 minutes to eliminate O₂ in the fermentation vial. The fermentation continued for 60 hours at 30 °C, and the medium was then stored at 4 °C for further analysis.

Results
Isolation of cellulolytic bacteria

Both aerobic and facultative anaerobic bacteria were isolated during the study. Their identities were obtained through 16S rRNA gene sequencing and summarized in table 1. One isolate from termite (TF2) resembled 100% sequence similarity to the bacteria *Brevibacillus brevis*, a cellulolytic bacterium that was also previously found in the termite *Zootermopsis angusticollis* [163]. Isolates SF1 and SF2 from sand sediments shared close sequence similarity to *Pseudomonas* sp.. Most facultative anaerobes were isolated from human feces samples (Table 1). FNP1 isolated from human feces samples shared 99% sequence similarity to *E. coli* and was a potential isolate for further fermentation study. Other facultative isolates belonged to *Enterobacter* sp. and *Klebsiella* sp. (Table 1).

Cellulase activity measurement

Four bacterial isolates (SF1, SF2, TF1 and TF2) that exhibited high cellulose hydrolysis ability on the Congo-Red agar plates were tested for their ability to hydrolyze cellulose filter paper in the basal salt media. A 12-hour culture from a single colony inoculation revealed high cellulose hydrolyzing ability from these isolates, whereas the control shown minimal change in the appearance of the cellulose filter paper (Fig. 1). SF1 isolate showed highest hydrolysis ability followed by TF2 (Fig. 1). The culture media was further tested for the glucose concentration by HPLC (LOD of 0.5 mM). However, the glucose concentration was not detectable in the hydrolysis media using this method (Fig. S1). A further step using LC-MS was taken to confirm whether glucose concentration was either really low or completely absent in the hydrolysis media. The LC-MS showed control level of glucose concentration in both SF1 and TF2 hydrolysis
media (Fig. S2), indicating that glucose was either quickly consumed after its production from cellulose hydrolysis or the efficacy of cellulose hydrolysis to glucose was limited by the critical enzyme β-glucosidase.

Fermentation ability by facultative anaerobes

The cellulolytic isolate FNP1 showed close sequence similarity to *E. coli* (Table 1), making it a valuable candidate for future study on consolidated bioprocessing (CBP). FNP1 was thus used to test its fermentation ability on the carboxymethyl cellulose (CMC)—the soluble form of cellulose. After 60-hour fermentation using CMC as the sole carbon source, the formic acid concentration was increased and acetic acid was produced in the fermentation media (Fig. 2). No ethanol was detected during this fermentation. Further study is necessary to discern which metabolic pathway was utilized for the CMC fermentation.

Discussion

The historically low demand for biologically-based transportation fuels is no longer the case [25]. The pressing desire to look for sustainable alternatives to fossil fuels has become increasingly high in priority. The high energy content of biologically derived fuels and their significant compatibility with existing petroleum-based transportation infrastructure, has made it even more attractive compared to other energy resources [25, 75]. Biofuel production from lignocellulosic biomass represents one of the most promising alternative fuel sources [1], and has drawn considerable interest over the past decade [13, 60, 77-79].

Lignocellulosic biomass, mainly consisting of cellulose, hemicellulose and lignin, is available on our planet in various forms including agricultural residues, forestry and
paper wastes and energy crops [15, 25]. However, the lignocellulosic biomass is naturally resistant to microbial and enzymatic degradation, known as biomass recalcitrance [15]. As a unique approach to developing biofuel from lignocellulosic biomass, consolidated bioprocessing (CBP) requires a single microbe that can facilitate both the saccharification and fermentation processes [35]. Metabolic engineering enables model organisms, like *E. coli* and *Saccharomyces cerevisiae*, to express the hétéologous cellulase needed to hydrolyze cellulose and other polysaccharides to monosaccharides, and to further ferment them into desired end products—from ethanol, butanol, to more advanced biofuels [2, 164]. However, the inability of yeast to process and release the recombinant cellulase at high rates limits the overall conversion process in CBP [44]. Similarly, microbes that secrete cellulase naturally are generally problematic in terms of their further development due to the lack of appropriate molecular biological tools as compared to model organisms [44]. The bacterium *C. thermocellum* is one of the most characterized anaerobic bacteria for the purpose of CBP, yet many unanswered questions remain [165]. The lack of microbes capable of efficiently releasing monosaccharides from the recalcitrant biomass is an obstacle that must be overcome in order for the full potential of CBP to be realized. The cellulosic biomass is naturally degraded by communities of microbes in various environments, including the gut of humans and termites, forest soils, or decaying matters [163, 166, 167]. Nature holds great potential for discovering candidates for CBP development. This study attempted to isolate microbes from various environments in order to select those that facilitate CBP, thus obtaining valuable candidates for use in the biofuel production. One candidate, FNP1, was isolated from human feces samples and resembles an *E. coli* strain with a sequence similarity of 99% (Table 1). The preliminary
study showed that it can ferment CMC and produce acetate and formic acid after 60-hour fermentation (Fig. 2). To our knowledge, this is the first cellulolytic E. coli isolate from a natural environment. Although the metabolic pathway behind the direct fermentation of CMC remains unclear and no ethanol was produced in the fermentation media, this organism presents itself as a great candidate for desired end product production via genetic modification. A previous study on Lactococcus lactis presented an excellent example of metabolic engineering for this purpose, in which the lactate dehydrogenase deficient cells can exclusively convert acetate to ethanol in order to maintain the redox balance [168]. The successful isolation of FNP1 should largely attribute to the direct enrichment of fresh feces samples immediately following collection. The 16S rRNA gene sequence of FNP1 showed 100% similarity to an uncultured human gut bacterium, further indicating its origin from the human gut where dietary fibers consisting of cellulose and hemicellulose are present. Other factors including sample source and enrichment carbon source may also have contributed to the successful isolation. FNP1 was obtained from only one of the two human feces samples in this study, indicating the variation of gut microflora in different people. Two different carbon source of cellulose paper and cellulose microcrystalline were used in the enrichment media, where the cellulolytic FNP1 was only isolated from the cellulose paper enrichment media, indicating the substrate preference by different cellulases.

Another approach of developing CBP organisms is to use the recombinant strategy where model organisms capable of producing desired end products are genetically engineered to express heterologous cellulase. The isolation of aerobic cellulolytic bacteria could supply potential microbial candidates for this purpose. One
obstacle involved in the development of CBP \textit{E. coli} is its inferior ability to secrete high quantities of cellulase in the media for hydrolyzing the lignocellulosic biomass [169]. One isolate in this study, TF2, showed 100\% 16S rRNA gene sequence similarity to a cellulolytic bacterium \textit{Brevibacillus brevis} that was previously isolated from the gut of termite \textit{Zootermopsis angusticollis} [163]. \textit{B. brevis} is known to secrete large quantities of proteins in the culture media [170] and thus could be a valuable candidate for engineering purposes in terms of secreting large quantities of cellulase enzyme in the culture media.

The aerobic bacterium TF2 showed consistent hydrolysis zones on the Congo-Red cellulose agar plate, and the overnight culture in basal media induced high amount of cellulose paper degradation (Fig. 1). In order for the cellulose to be completely hydrolyzed, three enzymes in the cellulase system have to be present and work coordinately. Endoglucanase and exoglucanase can respectively cut the cellulose backbone from inside and end of the polymers, releasing cellobiose that can be further hydrolyzed to glucose by β-glucosidase [171]. However, no glucose was detected by HPLC in the overnight TF2 culture media in this study. Previous studies indicate that cellulase activity was largely dependent on the efficient expression of β-glucosidase and that improved cellulase activity could be achieved by supplemental β-glucosidase [172]. Therefore, the undetectable glucose by HPLC in the culture media may be attributed to the inferior β-glucosidase ability of TF2 cellulase system. The detection of β-glucosidase by API-ZYM kit confirmed that no constitute β-glucosidase was detected in the TF2 culture. The high degree of cellulose filter paper degradation by TF2 thus indicated the presence of efficient endo- or exoglucanase in the TF2 cellulase system, making this bacterium a potential candidate for supplying these enzymes.
A realized CBP platform for lignocellulosic biomass derived biofuel production can benefit largely from the development of an efficient microbe whose capacity is not limited by either the saccharification or fermentation process. This study certainly supplied several candidate bacteria for this purpose.
Figure 1 Qualitative measurement of cellulase activity of various cellulolytic isolates (a. TF1, b. TF2, c. SF1, d. SF2 and e. uninoculated control) on the hydrolysis of cellulose filter paper.
**Figure 2** Fermentation of CMC by the cellulolytic bacteria FNP1.
Figure S1 Glucose detection by refractive index detector (RID) mounted to HPLC system. The retention time for glucose is 18.77 minutes. (a) Hydrolysates from TF2 hydrolysis media, (b) Hydrolysates from SF1 hydrolysis media.
Figure S2 Glucose concentration measurement by LC-MS in the hydrolysates of cellulolytic bacteria SF1 and TF2. The retention time for glucose is 13.8 minutes. (a) $10^{-6}$ M glucose control (b) glucose concentration in the SF1 hydrolysates (c) glucose concentration in the TF2 hydrolysates.
Table 1 Cellulolytic bacteria isolated from soil, sea sediments, termites and human feces.

<table>
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<tr>
<th>ID</th>
<th>Gas Requirement</th>
<th>Blast Results (NCBI Accession #)</th>
<th>Query Coverage</th>
<th>Max Identity</th>
<th>Isolation Source</th>
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<td>Aerobic</td>
<td><em>Sphingobacterium multivorum</em> (FJ459994)</td>
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<td>99%</td>
<td>Termite</td>
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<tr>
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<td>99%</td>
<td>Termite</td>
</tr>
<tr>
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<td>Aerobic</td>
<td><em>Pseudomonas geniculata</em> (HQ696469)</td>
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<td>99%</td>
<td>Sand Sediment</td>
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<tr>
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<td>Sand Sediment</td>
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<tr>
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<td>100%</td>
<td>99%</td>
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PART II

DYNAMICS OF PLANKTONIC FUNGI IN THE WEST PACIFIC

\[1\] This part of work is being submitted for journal publication. This dissertation will be delayed for access in ProQuest to avoid potential copyright conflict. The journal it publishes in holds the final copyright.
Abstract

Fungi contribute substantially to biogeochemical cycles of both terrestrial and marine habitats by decomposing matter and recycling nutrients. Yet, the diversity of their planktonic forms in the open ocean is poorly described. Using culture-independent approaches on samples from a broad swath of the Pacific Ocean across major environmental gradients, we found that planktonic fungi are molecularly diverse and these patterns were related to major phytoplankton taxa and various nutrients including nitrate, nitrite, orthophosphate and silicic acid. Over 400 fungal phylotypes were recovered across this region and nearly half of them grouped into two major novel lineages with Ascomycota and Basidiomycota as the dominant abundant groups at majority of the stations. These results suggest that planktonic fungi are a diverse and integral component of the marine microbial community and should be included in future marine microbial ecosystem models.
Introduction

The current ‘microbial loop’ hypothesis depicts microbes as a central player of marine matter and energy fluxes [173]. Molecular taxonomy and ecological genomics have revealed the efficiency with which organic carbon is processed by marine microbes in the surface waters of the ocean [174]. Specifically, heterotrophic bacteria and archaea are found to be largely involved in carbon and nutrient cycling in both coastal and oceanic waters [174, 175]. Although heterotrophic eukaryotic microbes are well documented in the oceans, their diversity and function remain relatively unknown. Particularly, large populations of planktonic fungi (i.e., filamentous free-living fungi and yeasts, and those associated with planktonic particles or phytoplankton) have long been known to exist in coastal and oceanic waters [176, 177], but their diversity and ecological function are still one of the most under-studied microbial topics.

Fungi are a key component of the biosphere, performing a wide range of biogeochemical and ecological functions across disparate environments [178, 179], and are particularly well-known for their important role in processing detrital organic matters from plants [173]. Marine yeasts have long been known to be ubiquitous in seawater [180-182]. A typical milliliter of seawater contains over several thousands of fungal cells (or propagules) [183] and evidence also revealed living fungal mycelia in seawater [184]. In freshwater ecosystems, fungal biomass can account for as much as 18-23% of the total mass of detritus [185-187]. Recently, fungal mycelia was found to be present as individual filaments or aggregates in the coastal upwelling ecosystem off central Chile [188] and the Hawaiian coastal waters [184] using the Calcofluor White stain method. These filaments or aggregates can reach up to 20 mm in diameter and over 50 mm long.
and are comparable to fungal mycelia detected in deep-sea sediments [189] and water-stable aggregates associated with mycorrhizal fungi in soils [190]. In seawater, organic aggregates represent a major growth habitat for planktonic microbial communities, and thus the combined metabolic activities of fungi and prokaryotic microbes can promote a highly efficient conversion of particulate organic matter (POM) to dissolved organic matter (DOM) in seawater [188, 191]. Furthermore, fungal biomass and diversity displays spatial and temporal variations in the coastal marine ecosystems [177, 188]. During the summer, fungal biomass in the coastal water can reach up to ~6 mg C/L of surface seawater [188] and is associated with an increase in phytoplankton biomass [192]; The vertical profile of fungal biomass and diversity concurred with those of primary productivity and/or physical parameters of the water columns (e.g., temperature and oxygen) [177, 188]. In the coastal water, high fungal biomass (hyphae) during active upwelling periods has been reported to be comparable to that of prokaryotes (bacteria plus archaea) [192]. Clearly, planktonic fungi are an important microbial component in the coastal marine ecosystems and, like other heterotrophic microplankton, are active in the water column and responding to primary production activity and organic matter availability [184, 188, 192].

In our previous study, we observed an interesting novel diversity of planktonic fungi and their interesting relationship with biological and physical features of seawater in the Hawaiian coastal waters [177]. Here, we focus on the diversity and ecology of mycoplanktonic communities in the oceanic waters, i.e., the Western Pacific, with the intention of answering the following questions. What is the overall fungal diversity distribution in the open ocean? Is certain phylum of fungi dominating certain
geolocations? How is their diversity and abundance partitioned across relatively large marine ecosystem in the open ocean? How does their diversity correlate with other players involved in marine matter and energy flux in the ocean?

**Materials and methods**

*Sample collection*

Seawater samples were collected from 5 depths (5m, 25m, 50m, 75m and 100m) of 6 geolocations (Station 2 (N19/E-160), Station 6 (N10/E-170), Station 10 (N0/E-180), Station 14 (N-9/E170), Station 20 (N-25/E165) and Station 24 (N-36/E162)) with the standard sampling rosette mounted around the CTD sensor package in the research cruise from Honolulu, Hawaii to Brisbane, Australia during January-February 2007 (Figure S1). Vertical profiles of fluorescence were measured using a Turner Designs 10-AU fluorometer [193]. The concentration of bacteria, *Prochlorococcus*, *Synechococcus* and picoeukaryotes were measured using flow cytometry according to the methods described [194]. A modified Becton Dickinson FACSCalibur flow cytometer was used to quantify all samples. The characteristic flow cytometric signatures of bacteria, *Prochlorococcus*, *Synechococcus*, picoeukaryotic phytoplankton were applied in gating these populations following standard population gating schemes [195]. The water samples for measuring concentrations of PO$_4$$^-$$^-$, NO$_3$$^-$$^-$, NO$_2$$^-$$^-$, and silicate were collected in a trace metal clean rosette, filtered through 0.4 μm filter membranes and frozen at -20°C for further analysis. Dissolved PO$_4$$^-$$^-$, NO$_3$$^-$$^-$ and NO$_2$$^-$$^-$ concentrations were measured according to the methods described by Hynes et al [196]. Silicate was measured following the method described by Strickland and Parsons [197].

*DNA extraction and library construction*
200 ml of each seawater sample was sequentially filtered through 2-micron and 0.22-micron polycarbonate filter membranes. The total genomic DNA was extracted from these two membranes using the FastDNA kit (Qbiogene, Irvine, CA) and was used as a PCR template for the amplification of the internal transcribed region (ITS) between the ribosomal RNA genes using the nested PCR approach following the procedure described by Gao et al. [183]. More specifically, highly conserved fungal rRNA gene primer ITS1F and primer ITS4 [198] were used in the first round of PCR reaction, and 1 µl of the PCR product in the first round was used as the template DNA for the nested PCR performed using primer set ITS3 and ITS4. All PCR reactions were performed following standard PCR protocol in 50 µl reactions for 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. PCR products from three separate amplification reactions were combined and purified using Gel and PCR Clean-Up Kit (Promega, Madison, WI). The purified PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI) and transformed into E. coli DH5α competent cells. Total of 30 (5 depths × 6 stations) ITS cloning libraries were constructed. For each library, plasmids carrying insert of correct size were sequenced using the T7 primers. Sequences obtained in this study were deposited in GenBank under accession numbers JX269176 - JX270134.

Sequence analysis

Sequences obtained in this study were aligned using Muscle v3.8 [199]. The aligned sequences were imported into Mothur v1.25 [200] for further quality control, trimming to the same lengths for further diversity analysis. Sequence identifications were done using the NCBI BLAST and all sequences with 99% sequence identity were
assigned into operational taxonomic units (OTUs) in Mothur [200]. Maximum likelihood calculation was done in PhyML v3.0 [201] corrected by model HKY85. The best tree was returned by searching tree topology using subtree pruning and regrafting (SPR) algorithm using 5 random starting trees.

*Diversity comparison*

The diversity within each fungal community (alpha diversity) was assessed by plotting rarefaction curve or calculating inverse Simpson index [200]. The similarity comparison between two communities (beta diversity) was performed based on UniFrac metric [202]. UniFrac distances are calculated based on the fraction of branch length shared between two communities in a phylogenetic tree constructed for all compared fungal communities. Mainly weighted Unifrac was employed in this study to assess the structure (membership and abundance) of fungal community. Principle coordinates analysis (PCoA) based on Unifrac distances was employed [200] to determine the major factors that separate fungal communities. The overall diversity comparison among all 30 fungal communities (5 depths × 6 stations) was generated into a heatmap based on the weighted UniFrac distances [200]. Hierarchical clustering was conducted using Unifrac distances based on the unweighted pair group method with arithmetic mean (UPGMA).

*Quantitative PCR*

Quantitative PCR was employed to assess the abundance of two major phyla of Ascomycota and Basidiomycota in all sampling sites. The fungal abundance was calculated by comparing to a standard curve plotted with cycle threshold (Ct) against DNA concentration. The concentration gradients of 0.01, 0.1, 1, 10, 100 μg/μL from the known Ascomycota and Basidiomycota DNA were applied for generating the standard
curve. Ascomycota and Basidiomycota DNA were amplified using the highly conserved fungal rRNA gene primers (ITS1F, ITS4-Asco and ITS4-Basidio) [198]. PCR reactions (20 μL) contained 10 μL of KAPA SYBR® FAST Universal 2X qPCR Master Mix (Kapa Biosystems), 1 μL of template DNA, 0.5 μL of 10 μM ITS1F, 0.5 μL of 10 μM ITS4-Asco or ITS4-Basidio, and 8 μL of water. The qPCR protocol was set up with an initial 3 min denaturation at 95 °C, followed by 40 amplification cycles of 95 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min. All PCR reactions were done in triplicate. The statistical significance (p<0.05) of Ascomycota and Basidiomycota abundance comparison between stations was determined by one-way analysis of variance (ANOVA) using untransformed data. The paired t-test (p<0.05) was performed to determine the difference between the Ascomycota and Basidiomycota abundance within each station.

Results and Discussion

Fungal diversity and major phyla abundance in the open ocean

To assess the abundance and diversity of planktonic fungi in the open ocean, we collected seawater samples from five depths in the euphotic zone from stations across an open ocean transect from the Hawaiian coast to Australia (Figure S1) across multiple environmental gradients. Clone libraries of the internal transcribed region (ITS) between the ribosomal RNA genes were generated for a total of 959 clones from 30 libraries. At 99% similarity these sequences represented 411 distinct fungal phylotypes among different stations (Table S1 and S2). Such a high diversity of planktonic fungi from the Western Pacific Warm Pool was reported for the first time in the oceanic waters. In comparison with earlier reports [177], the diversity of this study was much higher than that of Hawaiian coastal waters. The diversity discrepancy clearly ascribed to different
diversity analysis methods (clone library vs DGGE) and primers used in these two studies, which have been reported relating to the outcomes of various diversity analyses [179, 183].

Phylogenetic analysis indicated that the majority of these fungal phylotypes belonged to two major phyla of Ascomycota and Basidiomycota with highly diversified fungal lineages within these individual phyla (multiple branches) (Figure 1). Similar findings were reported in a recent review in which the authors collected broad data sets of fungal small subunit ribosomal DNA (SSU rDNA) and the phylogeny analysis revealed the abundance of these two phyla [203]. In this study, the phyla Ascomycota was found with sequences affiliating with subphyla Pezizomycotina and Saccharomycotina, whereas sequences of Basidiomycota belonged to three subphyla i.e. Agaricomycotina, Pucciniomycotina and Ustilaginomycotina. The phyla Ascomycota was found to be most abundant in the coastal regions of the Pacific Ocean islands (Station 2/N19E-160 and Station 24/N-36E162) (p=0.003), even though the two coastal stations are separated by thousands of kilometers (Figure 2). Conversely, Basidiomycota was found to be present across all investigated sampling sites with comparable abundance (p=0.587). Comparing between these two phyla, Basidiomycota abundance was dominating among all stations (p=0.005) (Figure 2). In addition to these two major phyla, we recovered two additional clades that have not been previously described in marine fungi (Figure 1 and Table S3). These two clades are deeply branching and did not show any significant similarity with the known sequences of the existing database. This discovery indicates the presence of a largely unknown fungal community in the pelagic ocean whose roles in the microbial food web and biogeochemical cycles are yet to be discovered.
Phylogenetic affiliation of fungal community

The majority OTUs (96.8%; 150 OTUs) of ascomycota belonged to the subphylum Pezizomycotina and only 5 of ascomycota OTUs derived from Australia coast (Station 24) were closely affiliated with the subphylum Saccharomycotina. OTUs of the subphylum Pezizomycotina belonged to 3 classes (Eurotinomycetes, Dothiodeomycetes, and Sordariomycetes) and affiliated to 10 different orders (Table S3). The vast majority (91.2%; 52 OTUs) of the Eurotinomycetes OTUs were closely related to Aspergillus sp. or Penicillium sp. (99-100% similarity), and most of these fungal OTUs were obtained from two coastal stations (Station 2 & 24). The Dothiodeomycetes OTUs were affiliated with 3 fungal orders: Dothideales, Botryosphaeriales and Capnodiales, many of which have close affiliation with known fungal species such as Hortaea werneckii (15 OTUs), Diplodia sp. (10 OTUs), Cladosporium sp. (15 OTUs) etc. (Table S3). Finally, fungal OTUs of the class Sordariomycetes were members of 5 orders: Glomerellales, Hypocreales, Trichosphaeriales, Xylariales and Microascales. Majority of OTUs (67.8%) from this class were retrieved from coastal stations (Station 2 and 24) with the exception of 9 OTUs within the family of Nectriaceae that were mostly found in the open ocean stations (Table S3).

Basidiomycota consists of 3 subphyla with the highest fungal diversity found in the subphylum Agaricomycotina (Table S3). Many fungal OTUs were distantly related to known species (<90% sequence similarity), probably belonging to new species or new genus. For example, 3 fungal OTUs (S20D4-12/14/15) found in station 20 had less than 90% similarity to Phlebia acanthocystis. Twenty OTUs of the subphylum Pucciniomycotina have high similarity (close to 99%) to Rhodotorula mucilaginosa. In
addition, all 66 OTUs of the subphylum Ustilaginomycotina were found to have the highest similarity to *Malassezia* sp. (86%-99% sequence similarity), indicating the abundance of *Malassezia* sp. in the open ocean. Interestingly, our previous study also revealed the abundance of this species in marine sponges [183].

The majority of OTUs found in the two unknown groups in the phylogenetic tree could not be attributed to any known fungi or other eukaryote groups. More specifically, 122 OTUs of unknown group 1 were closely affiliated (80-85%) with an unknown fungus sequence derived from marine subsurface sediments (Table S3). OTUs of unknown group 2 (57 OTUs) were more diversified, belonging to unknown fungi or other eukaryotic sequences derived from marine environments. However, their identity could not be determined because of the low sequence coverage and similarity with known sequences in the NCBI database. Out of 57 OTUs, 48 couldn’t be affiliated with any known fungi or other eukaryote sequences when comparing their full length sequences. In addition, the other 9 OTUs were only affiliated with a known eukaryote sequences at the similarity level of around 80% (Table S3). Clearly, results of this study suggest largely undiscovered fungal species in the oceanic water.

**Fungal community structure and diversity comparison**

The difference of fungal community was assessed using UniFrac, a phylogeny-based metric [202]. Overall, the fungal community diversity was highest near Hawaii coast (Station 2/N19E-160) but similar among open ocean stations along the transect (Figure S2). This suggests the higher mycoplankton diversity in coastal water ecosystems than the open ocean and is also in concordance with the previous study [177]. Carbon from autochthonous primary production and allochthonous (terrestrially derived)
production is higher near the coast and this source [204] that exceeds the consumption of herbivores, along with elevated levels of detritus and other forms of nutrients [204], favors increased mycoplankton abundance and diversity. This organic detritus serves as nutrient sources for fungal component in these ecosystems [205], making these environments a rich source of diverse mycoplankton communities. Similar findings were also reported in research of culturable fungi, in which fewer fungal isolates were obtained in pelagic water samples than those from coastal waters [206]. The unweighted UniFrac revealed an overall distance of 0.74 (p<0.001) between two communities of all stations. The fungal community comparison based on weighted UniFrac further indicated slightly higher distance within stations compared to that of between stations (Figure S3), suggesting a complex vertical fungal community structure in the euphotic zone of specific geolocations. This was further supported by the hierarchical clustering where fungal communities from coastal stations (Station 2 and Station 24) were readily grouped but fungi from other stations were dispersed along a nested structure which seemed to follow the pattern of sampling order along the transect (Figure S4). The individual comparison between two fungal communities revealed overall high distances (Figure S5), suggesting an underrated and versatile role of fungi in various marine environments. Weighted UniFrac-based principal coordinate analysis (PCoA) separates fungal communities primarily by geolocations where open ocean stations were clustered together with coastal and equator communities stood out (Fig. 3A). This variation (PC1 of 29.8% and PC2 of 25.7%) by geolocation was not readily explained by all measured environmental factors like nutrients or other autotrophs and heterotrophs. Compared to other bacterioplankton, fungi arm themselves with unique enzymatic system for
hydrolyzing resistant long chain carbohydrate substrates [205]. A possible explanation for the variation thus could be their participation in the degradation of recalcitrant dissolved organic matters (RDOM) generated in the microbial carbon pump (MCP) [207]. However, further efforts on the elucidation of RDOM distribution and their degradation by fungi would be necessary to support this. The coordinates PC3 (16.9%) and PC4 (15.9%) further revealed variation reflected by nutrients (nitrate, nitrite and phosphate) level and other photosynthetic phytoplankton including picoeukaryotes and *Synechococcus* (Figure 3B and S6). This evidence thus suggests possible fungal consumption of dissolved organic matter (DOM) generated by these primary producers of ocean ecosystems and is also consistent with the report that the vertical distribution of fungal biomass closely related to that phytoplankton biomass in coastal water [192]. At these three high nutrient stations (Station 10, 14 and 24) where nitrogen was not limited, the highest primary production was achieved at shallower depths; the oligotrophic stations (Station 6 and 20), on the other hand, only reached the highest primary production at deeper depths when nutrients were available (Figure 4). Overall, the primary producers including *Prochlorococcus*, *Synechococcus* and picoeukaryotes showed a similar vertical pattern to chlorophyll profile in both coastal and open ocean stations albeit the discrepancy that one might be slightly dominating the primary production than others at specific depths (Figure 4). As irreplaceable food web components, marine microbes reveal their importance in the carbon cycling in the pelagic zone of oceans [208, 209]. Heterotrophic bacteria and fungi share the function of mediating carbon and nutrients flux in marine ecosystem and are tightly related [210]. Among all stations, concentrations of bacteria were mostly higher near surface and
decreases along depths, indicating their role in recycling and incorporating upwelling organic matter into higher food webs (Figure 4). Fungal OTUs followed a similar vertical pattern as bacteria, suggesting their involvement in the organic matter consumption (Figure 4). In addition, the abundance of Ascomycota and Basidiomycota at majority of the stations was relatively higher at surface and decreases along depths (Figure 2). Collectively, both the fungal diversity and major phyla abundance revealed a possible involvement of fungi in organic matter consumption in the euphotic zone of ocean ecosystems.

All together, our results show that planktonic marine fungi are molecularly diverse, including previously undescribed lineages, and that a variety of fungi could be participating in multiple biological processes including dissolved organic matter consumption. These results suggest that planktonic fungi are integral components of the marine microbial community, and are likely to be a versatile group involved in multiple biological processes and should be included in future marine microbial ecosystem models. Future multi-gene, genomic or transcriptomic analyses of fungal signatures of these open ocean ecosystems will help provide deeper insight into their molecular diversity and their functional role in pelagic ecology and biogeochemistry.
Figure 1 Maximum likelihood phylogenetic tree generated from 744 unique fungal sequences derived from 30 marine fungal clone libraries. Sequences are aligned using Muscle v3.8 and undergo quality control in Mothur v1.25 before used for phylogenetic tree generation. Maximum likelihood calculation is done in PhyML v3.0 corrected by model HKY85. The best tree was returned by searching tree topology using subtree pruning and regrafting (SPR) algorithm starting with 5 random trees.
Figure 2 Evaluation of Ascomycota and Basidiomycota abundance by quantitative PCR among depths of all stations. The abundance represented by their total DNA concentration in the original sample was calculated based on standard curves created using two known Ascomycota and Basidiomycota species, respectively. Station 2 (N19/E-160) and 24 (N-36/E162) were coastal stations near Hawaii and Australia, respectively; Station 10 (N0/E-180) was station at the Equator; Station 6 (N10/E-170), 14 (N-9/E170) and 20 (N-25/E165) were open ocean stations.
**Figure 3** Principal Coordinates Analysis (PCoA) of marine fungal community based on the weighted-UniFrac distance matrix. Fungal communities from different geolocations were indicated as different symbols. The percentages of variation at the first four major coordinates were indicated. A. Fungal community variation explained by coordinates PC1 and PC2; B. Fungal community variation explained by coordinates PC3 and PC4.
Figure 4 Fungal OTU number at various depths of each sampling station was plotted along with other biotic and abiotic factors. Fungal OTU (filled square), bacteria (filled circle), *Prochlorococcus* (filled triangle) and *Synechococcus* (open triangle), picoeukaryotes (inverse triangle) and chlorophyll (green line) were plotted against the sampling depths. At each depth, various nutrients including PO$_4^-$, NO$_3^-$, NO$_2^-$ and Silicic acid were indicated as colored bars.
Figure S1 CTD sampling stations in the Western Pacific during the cruise of R/V Kilo Manoa in June 2008. Station locations were indicated as the number in the white circle.
**Figure S2** Rarefaction curve for marine fungal diversity among 6 stations. Phylogenetic diversity is represented by branch length.
Figure S3 Mean (±SEM) weighted UniFrac distance of fungal communities within and between various stations.
Figure S4 Hierarchical clustering of fungal communities in various stations and depths. The bar indicates a weighted UniFrac distance of 0.1.
Figure S5: Heatmap showing the fungal diversity comparison among various stations and depths. The scale at the bottom of the heatmap indicates the similarity level between each comparison. The darker the color is, the more different the two comparing fungal communities are.
Figure S6 Distribution of various nutrients (PO$_4^-$, NO$_3^-$, NO$_2^-$ and Silicate), picoeukaryotes and *Synechococcus* among investigated sampling locations. For each station, the data was averaged from the measurements of 5 different depths representing the station abundance.

![Nutrients distribution](image1)

**B. Pico-eukaryotes distribution**

![Pico-eukaryotes distribution](image2)

**C. Synechococcus distribution**

![Synechococcus distribution](image3)
Table S1 Diversity indices for fungal communities from 5 depths of 6 different stations. For each fungal community, the sequencing numbers (Seqs#) and the obtained OTU number (OTUs) are recorded. The coverage of each library is calculated and the Inverse Simpson Index is calculated to indicate the estimated fungal diversity from each community.

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Table S2 Diversity indices for fungal communities from 6 sampling stations. Fungal data from 5 depths of each station were combined and treated as one large fungal community. For each fungal community, the sequencing numbers (Seqs#) and the obtained OTU number (OTUs) are recorded. The coverage of each library is calculated and the Inverse Simpson Index is calculated to indicate the estimated fungal diversity from each community.

<table>
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### Table S3 Phylogenetic affiliation of marine fungi.

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<th>Representative Species (accession no.)</th>
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<th>Major distribution</th>
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<td>Saccharomycetaceae</td>
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<td>Malasseziaceae</td>
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<td>Family</td>
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<td>Microbotryomycetes</td>
<td>Sporidiobolales</td>
<td>mitosporic Sporidiobolales</td>
<td><em>Rhodotorula mucilaginosa</em></td>
<td>(JX156378)</td>
<td>S6/14/20</td>
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<td><em>Sporobolomyces</em> sp.</td>
<td>(JQ936286)</td>
<td>1 (JX269238)</td>
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<td>mitosporic Filobasidiales</td>
<td><em>Cryptococcus</em> sp.</td>
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<td>Polyporaceae</td>
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<td>1 (JX269992)</td>
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<td>9 (JX269237)</td>
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APPENDIX

Bibliography


(2) Li Q, Wang X, Jiao N and Wang G. Abundance and novel lineages of Thraustochytrids in Hawaiian Waters. (Under review)


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197. Strickland JDH, Parsons TR (1972) A Practical Handbook of Seawater Analysis. Fisheries Research Board of Canada


