Metagenomic and Proteomic Analyses of a Mangrove Microbial Community Following Green Macroalgae Enteromorpha prolifera Degradation

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Introduction

Global energy demand is increasing, creating problems such as energy security, resources depletion, environmental degradation, and climate change, which are expected to increase over the next decades. Biomass is considered a sustainable renewable energy source. The biofuel potential in marine biomass is more than 100 EJ/year and significantly higher than the values for land biomass (22 EJ/year) [7]. Third-generation algae-based biofuel feedstock, seaweed, and macroalgae are considered to have potential for biofuel production. Algae biomass shows higher yields and rates, higher photosynthetic efficiency, and lower land requirements [1]. Furthermore, macroalgae have greater easily hydrolyzable carbohydrate and low lignin contents, enabling enzymes access to these substrates and achieve an efficient hydrolysis rate [7, 33].

Enteromorpha (Ulva) prolifera (Muell.) J. Agardh (Chlorophyta, Ulvales), which is distributed worldwide in the intertidal zone of the sea, is the dominant species of green tide (Fig. 1). Enteromorpha prolifera (EP) caused the world’s largest transregional macroalgal bloom in the Yellow Sea of China. The massive green tide covered 600 km², and more than one million tons of green alga were cleaned from the beach and coast [26]. In addition, Ireland, France, Italy, and Japan suffered from massive Ulva (Enteromorpha) green tides on the shoreline. EP dry matter is mainly composed of approximately 45% water-soluble carbohydrate, 15% crude
fiber, and 14% crude protein [22]. E. prolifera is a high-valued biomass rich in complex carbohydrate and fiber contents. One critical step in biofuel development is determining the most favorable conditions for enzymatic saccharification to hydrolyze the biomass to fermentable sugars. The saccharification of EP can be advantageous when EP is treated with a mixture of effective enzymes. However, the polysaccharides present in EP have not been fully characterized [53] and the complex structures are difficult to degrade. Mangrove ecosystems are very important for both maintaining and improving biological environments [25]. The humid subtropical climate fosters microbial diversity in mangrove sediments, and the microorganisms that thrive in mangrove environments are a valuable source of novel enzymes.

Metagenomics is a powerful approach for determining the structure, diversity, gene content, and functional composition of microbial communities. It also provides insight into the large number of uncultured microbes [9]. Next-generation sequencing (NGS), known as high-throughput sequencing, plays a key role in the analysis of shotgun sequencing-based metagenomics [16]. The four commercially available NGS platforms (Illumina/Solexa, 454/Roche, ABI/SOLiD, and Helicos BioSciences) have provided unprecedented opportunities for functional genomic research based on their ability to generate hundreds of megabases of data [32, 36]. The Illumina/Solexa Genome Analyzer is the most widely used sequencing platform in the field and currently dominates the NGS market [31]. Illumina constructed longer and more accurate contigs and scaffolds than other platforms did, and is a more appropriate approach for metagenomic surveys [27, 30, 37].

Metaproteomics is a new field within the “omics” sciences that attempts to identify all proteins expressed in a given ecosystem. Metaproteomic studies play an important role in the determination of microbial function in a variety of environments [45, 48–50]. The use of two-dimensional gel electrophoresis (2-DE) in combination with liquid chromatography (LC)-mass spectrometry (MS)/MS approaches has become widely used in recent studies. Recently, with the availability of extensive metagenomic sequences from various marine microbial communities, metaproteomics has attracted considerable attention in the marine biology field to answer a variety of questions. This method can also be used to determine specific features of mixed marine samples and overall microbial ecosystem function.

The biodegradation of different terrestrial biomasses with microbial communities has been well-documented [3], but the EP-degrading microbial communities from mangrove ecosystems are largely unknown. A better understanding of the EP-degrading bacterial communities can facilitate the development of the algal biofuels industry. The use of metagenomics and proteomics may play a key role in determining the molecular mechanisms by which microorganisms attack and degrade the green macroalgae (Fig. 2). Therefore, the purpose of this study was to investigate an EP-degrading community from mangrove sediment using an Illumina HiSeq 2000 sequencing system and LC-MS/MS. Our results provide insights into the biodegradation of green macroalgae.

Materials and Methods

Enrichment of EP-Degrading Microbial Community

Green macroalgae EP was collected from a beach (N36°03’, E120°20’) in Qingdao, China (Fig. 1). The soil sediments were sampled from a typical mangrove protection zone in the Dongzhai Port of Haikou, China. The mangrove sediment samples were enriched with the EP powders as the sole source of carbon and energy. An EP-degrading enrichment culture was obtained and named DZ21. The community studied was capable of digesting the green macroalgae. The EP-degrading samples after degradation, coated with a gold sputter coating, were dried for scanning electron microscopic (SEM) observation (JSM-6380, Japan).

Total DNA Isolation and Sequencing DNA on the Illumina HiSeq 2000 PE-100 Platform

Total microbial genomic DNA of the EP-degrading community was extracted as previously described [42]. The DNA concentration was determined with a NanoDrop 2000 Spectrophotometer and then analyzed by gel electrophoresis. A highly pure (A_{260}/A_{280} = 1.8) and large amount of total genomic DNA (200–360 ng/μl) was generated (Fig. S1). Total DNA from the bacterial community was sheared to produce a DNA fragment library for whole-genome shotgun sequencing according to the manufacturer’s instructions.
from the TruSeq DNA sample preparation kit (Illumina, USA). DNA sequencing was performed on an Illumina HiSeq 2000 sequencer, which generated 100 bp paired-end (PE) raw reads.

Statistics of Sequencing Data De Novo Assembly of Illumina GA Short Reads

The obtained raw sequences were submitted to NCBI’s Sequence Read Archive (SRA). The metagenomic sequences were deposited at the NCBI SRA under GenBank Accession No. SRX590675. After removal of adaptor sequences, ambiguous “N” nucleotides (ratio of “N” > 10), and low-quality sequences (Q-value ≤ 5) [24], clean reads were assembled by using the Short Oligonucleotide Alignment Program (SOAPdenovo) [23, 24]. Raw reads and contigs were used for further analysis.

Gene Prediction in Non-Redundant Microbial Gene Sets of Metagenomes

MetaGeneMark was used to predict open reading frames (ORFs) from the assembly contigs as well as merging contigs from the sample. Next, BLAST was used to align the predicted ORFs. A pair of genes with more than 95% identity over 90% of the shorter ORF length was grouped together. Groups sharing genes were then merged, and the longest ORF in each merged group was used to represent the group, whereas the other members of the group were considered redundant. ORFs with lengths less than 100 bp were discarded, and the qualified ORFs were translated.

**Fig. 2.** Schematic overview of the application of metagenomics and proteomics to study the Enteromorpha prolifera-degrading microbial community.
into amino acid sequences using the NCBI’s Genetic Codes.

Gene Taxonomic Assignment and Gene Functional Classification

The taxonomic classification of metagenomic reads was performed using the BLAST-based tool against the NCBI non-redundant (nr) protein database [4] with an E-value cut-off of 1e-5. Species richness and diversity analysis based on sequencing reads and assembled contigs were determined using MEGAN. The MEGAN program uses a lowest common ancestor algorithm based on their blast bit scores to classify reads to indicated taxa [13]. The lowest common ancestor parameters used were min-support 5, min-score 35.0, and top-percent 10 [24]. To investigate the gene profile characteristics of the EP-degrading community, all sequencing reads were functionally annotated against the Clusters of Orthologous Groups of proteins (COGs) database [43]. COGs were identified according to BLASTX analysis with an E-value cut-off of 1e-5. All candidate regions with a query coverage ≥50% were retained [39]. The final sequencing reads were assigned to the COG functional categories based on their best BLAST hits. Gene annotation was predicted in the orthology databases of KEGG (Kyoto Encyclopedia of Genes and Genomes) and eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups). The predicted and known protein functions of COGs were classified into 23 categories. Using the NC-IUBMB Enzyme Commission (E.C.) nomenclature, enzyme data and metabolic pathway information were obtained by downloading relevant maps from the KEGG database.

Bacterial Protein Extraction and Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

The time-course dynamics of cellulose degradation by community DZ21 was determined and the crude fiber had clearly degraded after 7 days. The whole proteins of community DZ21 after treatment for 1 day (D1) and 7 days (D7) were analyzed by a combination of 2D-PAGE and LC-MS/MS. A total of 50 mg of cells was dissolved in 5 ml of ice-cold 20 mM Tris·HCl buffer (pH 8.0) consisting of 1 mM KCl, 2 mM MgCl2 and 10 mM dithiothreitol (DTT), and then sonicated for 5 min with 2 sec ON and 3 sec OFF at 180 W, three times. Next, nuclease (v/v, 100:1) was added to the sample followed by incubation at 37°C for 30 min. Subsequently, 10% (w/v) trichloroacetic acid in ice-cold acetone solution was added at a ratio of 20:80 (v/v), and placed at −20°C for 2 h in order to improve protein precipitation. The pellet was centrifuged at 20,000 xg for 45 min (4°C) and washed three times with ice-cold solution. The supernatants were removed and 500 µl of lysis buffer consisting of 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and 10 mM DTT was added to the pellet. The suspension was sonicated for 5 min with 2 sec ON and 3 sec OFF at 180 W to improve protein solubilization. Finally, the samples were centrifuged at 20,000 xg for 30 min at 4°C and the resulting supernatant was subjected to isoelectric focusing. The total protein concentration was measured using the Bradford assay [5]. A volume of 125 µl, corresponding to 50 µg protein, was loaded onto 7 cm immobilized pH gradient (IPG) strips, pH 4–7. Isoelectric focusing was then performed at 20°C with a current setting of 50 mA/strip as follows: the IPG strip was rehydrated at 30 V for 12 h, and then proteins were focused successively for 1 h at 500 V, 1 h at 1,000 V, and 8 h 20 min at 8,000 V. Next, the IPG strip was equilibrated in DTT and iodoacetamide buffer. For the second dimension, the proteins were run on a 12.5% SDS-polyacrylamide gel at a constant current of 17 mA. Protein spots were stained with Coomassie brilliant blue. All experiments were conducted in triplicates.

Gel Images, Statistical Analyses, and Protein in-Gel Digestion

Gel images were analyzed using Melanie 3.02 software (Genebio, Switzerland). Spot detection, spot measurement, spot matching, and background subtraction were performed. Only protein spots displaying reproducible change patterns and showing significant difference in up- or down-regulation, were considered to be differentially expressed proteins for further identification. Excised protein spots from 2D gels were digested as described by Oda et al. [34].

LC-MS/MS Analysis and Database Search

The target protein spots were analyzed by LC-MS/MS. The samples were then concentrated and desalted using a reversed-phase C18 precolumn, and further peptide separation was achieved using a nano-HPLC reversed-phase C18 column with an UltiMate nano-HPLC system and a 50 min linear gradient of acetonitrile. The column outlet was directly coupled to a Q Exactive Orbitrap (Thermo Fisher Scientific, USA) mass spectrometer working in the regime of data-dependent MS to MS/MS switching. The electrospray voltage and cone voltage were 1,500 V and 30 V, respectively. The obtained peptide data from the excised spots were analyzed automatically by database matching against the NCBI nr protein database using Mascot ver. 2.3 (Matrix Science, UK) [35].

Statistical Analysis

The normalized integral values were statistically analyzed using SPSS ver. 13.0 for Windows (SPSS, Inc., USA). One-way analysis of variance was used to determine the statistically significant differences in selected signals among the samples. A p-value of less than 0.05 was considered to indicate statistical significance.

Results

Isolation and Characterization of EP-Degrading Microbial Community from Mangrove Sediments

Samples showing degradation were observed by SEM. The untreated EP exhibited a rigid and ordered structure (Fig. 3A). After treatment with the EP-degrading community DZ21 for 7 days, the EP was severely decayed and contained more holes, cracks, and erosion troughs on the surface.
The composition changes in EP treated with the community were determined (Fig. S1). Total dissolved solids and total soluble sugar concentrations were reduced by 88.4% and 68.9%, respectively. The biological treatment of EP for 7 days led to 42.1% degradation of crude fiber. The yield of reducing sugar was 0.26 g/g dry EP, indicating high potential for industrial application.

**Sequencing and Metagenomic Assembly by SOAPdenovo**

Isolation of total community DNA using a CTAB-based extraction method with a gel filtration purification procedure yielded high-quality genomic DNA (Fig. S2). Clean “reads” were screened from raw sequencing reads after removing of adaptors, low-quality reads, and ambiguous reads. Approximately 65.9 million (6.59 G bp) clean reads were obtained. Metagenomic Paired-end Illumina sequence reads were assembled using SOAPdenovo software. This approach resulted in 17,138 large contigs with an average length of 1,838 bases and an N50 of 4,175 bp. When mapping single-end and paired-end reads simultaneously, the mapping rate was 62.5%.

**Microbial Community Composition**

To assess the species richness of this system, we used rarefaction analysis based on reads assigned to the NCBI taxonomy. The rarefaction curve was nearly horizontal at 100% of the total reads, indicating that the DZ21 library was close to saturation. The reads assigned to the kingdom Bacteria accounted for 55.02% of the total reads, whereas 35.56% of the total reads showed no hits in the present database. Based on taxon identification analysis, the most prevalent bacterial taxa at the phylum level were Proteobacteria (54.26% of hit-bacterial reads), Bacteroidetes (0.35% of hit-bacterial reads), and Actinobacteria (0.19% of...
hit-bacterial reads). A small number of reads was assigned to Firmicutes, Deinococcus-Thermus, and Streptophyta (Fig. 4A). At the genus rank, Novosphingobium (11.24% of hit-bacterial reads) was the prevalent genus in this community. In addition, Sphingopyxis (7.92%) and Pseudomonas (3.37%) were also present (Fig. 4B). Following the most predominant species Pseudomonas stutzeri (53.07%), the top five species were Sphingopyxis alaskensis (16.91%), Ochrobactrum intermedium (12.56%), Ochrobactrum anthropi (3.42%), and Bordetella sp. (2.89%).

Gene Function Annotation and Classification

Algae and land plants differ in cell wall composition. The key difference is the low concentration in the presence or absence of lignin in macroalgal feedstocks. The chemical structures and contents of carbohydrates in green algae and land plants also differ significantly. From the perspective of biomass conversion, appropriate enzymes must be introduced to liberate biomass for hydrolysis. The composition of the enzymatic mixture largely depends on the biomass origin. As a potential biomass material, EP has practical values in the biomass energy, food, and medicinal industries. To obtain a metabolic profile of community DZ21, all contigs were annotated using the BLASTX tool based on the COG, KEGG, and CAZy databases. The genes annotated by the COG database were classified into 23 COG functional categories. A large number of contigs were classified as “function unknown (S),” “energy production and conversion (C),” “amino acid transport and metabolism (E),” “carbohydrate transport and metabolism (G),” and “inorganic ion transport and metabolism (P)” (Fig. 5). According to the BLASTX results of total contigs against the CAZy database, we detected 22 homologous genes classified into 13 different glycoside hydrolase (GH) families, mainly belonging to GH13, GH51, GH10, GH1, GH53, and GH43, based on the contig aligned number (Table S1). In the GH family, enzymes involved in the hydrolysis of cellulosic materials were detected, including cellulase (E.C. 3.2.1.4), beta-1,4-xylanase (E.C. 3.2.1.8), beta-glucosidase (E.C. 3.2.1.21), alpha-L-arabinofuranosidase (E.C. 3.2.1.55), and alpha-glucuronidase (E.C. 3.2.1.139) (Table S2). Most were found to belong to Pseudomonas.

Two-Dimensional Electrophoresis and Identified Proteins

The metaproteome profiles of the microbial community were detected by two-dimensional gel electrophoresis (Fig. 6). The use of pH 4–7 IPG strips resulted in well-spread protein spots, which enabled accurate excision and image identification. All spots were separated and showed relative molecular masses of 19–99 kDa (Fig. 6). A total of 20 relevant protein spots were excised from the stained 2D gel and digested with trypsin, and the collected peptides were analyzed by MS/MS. These protein spots were identified and further characterized to determine their

![Fig. 5. Clusters of orthologous groups (COG) functional classification of the community DZ21 metagenome.](image-url)
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correlations with bioinformatic databases. The peptide mass peaks were compared with those in the NCBI database. The protein identification data, including GenBank ID, molecular weight, isoelectric point value, Mascot score, and function are listed in Table 1. All digested spots present in the gel were evaluated in triplicates.

Discussion

During the erosion process, mangrove-degrading bacteria affixed themselves to the surface of the matrix and colonized on the surface to form a strong and compact network over the exterior surface, after which the bacteria secreted extracellular enzymes to degrade EP. This may be the main cause of EP degradation. Deswal et al. [8] employed crude enzymes for saccharification of straws, and the enzymolysis reducing sugar yields of wheat straw and rice straw as substrates were 0.214 and 0.157 g/g dry mass, respectively. As expected, the biomass conversion rate was higher because of the low contents of hemicellulose and lignin.

NGS technology offers a new and rapid approach for genome-wide analysis. To characterize microbial community DZ21, including its genome structure, gene content, metabolic capabilities, and role of specific organisms for EP degradation, bacterial sequences within the metagenomic data were identified using the Illumina HiSeq 2000 sequencing technology. The microbial composition of DZ21 was analyzed based on mapped reads using MEGAN. It is well known that one bacterial community shows greater efficiency compared with a single strain in the biodegradation of biomass materials [53]. However, little information is available regarding macroalgae degradation in natural environments, particularly mangrove environments. Novosphingobium species are an important source of cell wall polysaccharide-degrading enzymes such as rhamnosidase [14]. Moreover, the water-soluble polysaccharide of EP contained mainly rhamnose, galactose, and xylose [52]. In addition, Novosphingobium was reported to affect lignin biodegradation [6]. Most Sphingopyxis species are capable of expressing lignocellulolytic glycoside hydrolases [40, 41]. There are previous reports of Pseudomonas strains with fiber- and polysaccharide-degradation abilities. Pseudomonas sp. has been reported to degrade lignin [38]. Bacterial laccase from P. stutzeri has been employed in lignocellulosic fibers [20]. Additionally, Maalej et al. [28, 29] demonstrated the production and biochemical characterization of a high maltotetraose-producing amylase from P. stutzeri. Ochrobactrum species (α-Proteobacteria) were found to be present in the gut of the wood-feeding higher termite Zootermopsis angusticollis [47]. There are also reports of Ochrobactrum strains with lignin degradation ability [44]. Bordetella is beneficial in oxygen consumption and pH neutralization in the cellulose-degrading community [17, 18]. Bordetella is generally considered to be independent of the substrates derived from cellulose degradation, such as saccharides. Our results are similar to those of previous reports and indicate that bacteria of Sphingopyxis and Ochrobactrum are key EP degraders and function together with other degrading bacteria in the mangrove community. Prospecting macroalgae as biomass for bioconversion is limited primarily by the

Fig. 6. Two-dimensional gel electrophoresis of proteins extracted from D1 (A) and D7 (B). The proteins were separated on a 7 cm immobilized pH gradient strip with a linear gradient of pH 4–7. Numbered circles indicate spots showing an expression level change between D1 and D7. The proteins are listed in Table 1.
availability of tractable microorganisms that can metabolize alginate [46]. Enzymatic treatment of algae has been reported for *Laminaria* and was successfully saccharified using an enzymatic mixture.

The annotation and classification of functional genes is very important for understanding the mechanism of community DZ21 degradation. The types and contents of heteropolysaccharides from algae are largely species-dependent. Based on previous studies, most *Pseudomonas* species are capable of fermenting various carbohydrates. However, the involvement of *Pseudomonas* species in EP degradation has not been reported and their function in EP degradation remains unknown. Although genetically identical, the species possess significant discrepancies and can adapt under different fermentation conditions [21]. The bottleneck of biofuel production is the effective conversion

### Table 1. Proteins originating from microorganisms identified by MALDI TOF/TOF MS.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein</th>
<th>Gene ID in NCBI</th>
<th>Mascot score (MS/MS)</th>
<th>Matching peptides</th>
<th>Best-matched organism</th>
<th>Theoretical MW kDa</th>
<th>Theoretical pI</th>
<th>Expression level</th>
</tr>
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<tbody>
<tr>
<td>108</td>
<td>ATP synthase subunit beta</td>
<td>119386525</td>
<td>117</td>
<td>2</td>
<td><em>Paracoccus denitrificans</em> PD1222</td>
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<td>298</td>
<td>ATP synthase subunit beta</td>
<td>77964181</td>
<td>192</td>
<td>5</td>
<td><em>Clostridium paradoxum</em></td>
<td>50.46</td>
<td>4.99</td>
<td>↑</td>
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<tr>
<td>311</td>
<td>Elongation factor complex EF-TuEF-Ts</td>
<td>1942721</td>
<td>97</td>
<td>2</td>
<td><em>Escherichia coli</em></td>
<td>42.18</td>
<td>5.22</td>
<td>↓</td>
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<tr>
<td>320</td>
<td>Hypothetical protein NAP1_12578</td>
<td>85709719</td>
<td>273</td>
<td>15</td>
<td><em>Erythrobacter</em> sp. NAP1</td>
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<td>5.03</td>
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<td>328</td>
<td>Superoxide dismutase</td>
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<td>22.34</td>
<td>4.89</td>
<td>↑</td>
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<tr>
<td>335</td>
<td>Elongation factor Tu</td>
<td>85374468</td>
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<td>3</td>
<td><em>Erythrobacter litoralis</em> HTCC2594</td>
<td>43.06</td>
<td>4.80</td>
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<tr>
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<td>H(+)-transporting ATP synthase beta chain</td>
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<td>8</td>
<td><em>Flavobacterium</em> bacterium BBFL7</td>
<td>54.12</td>
<td>4.81</td>
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<td>369</td>
<td>Transcriptional regulatory protein</td>
<td>86144202</td>
<td>364</td>
<td>17</td>
<td><em>Leeuwenhoekiella blandensis</em> MED217</td>
<td>27.89</td>
<td>5.25</td>
<td>↑</td>
</tr>
<tr>
<td>395</td>
<td>Enoyl-(acyl-carrier-protein) reductase</td>
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<td><em>Marinobacter</em> sp. HTCC2170</td>
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<td>5.09</td>
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<td>34</td>
<td><em>Roseovarius</em> sp. 217</td>
<td>42.97</td>
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<tr>
<td>638</td>
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<td>85709060</td>
<td>595</td>
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<td><em>Erythrobacter</em> sp. NAP1</td>
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<td>4.80</td>
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<td><em>Gamma proteobacterium</em> HTCC2080</td>
<td>50.12</td>
<td>4.71</td>
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of algal feedstocks into glucose [2]. In our study, important enzymes of the metabolic pathways involved in biodegradation were detected in association with *Pseudomonas* in KEGG pathway analysis, such as xylan 1,4-beta-xylosidase (E.C. 3.2.1.37), alpha-N-arabinofuranosidase (E.C. 3.2.1.55), pectate lyase (E.C. 3.1.1.11), cellulase (E.C. 3.2.1.4), beta-glucosidase (E.C. 3.2.1.21), and beta-mannanase (E.C. 3.2.1.4), indicating that *Pseudomonas* species likely contribute to EP degradation during the fermentation (Table S2).

In the analysis of the obtained proteome, the proteins identified in the metaproteome of community DZ21 had diverse functional activities related to glycolysis, protein biosynthesis, oxidation-reduction, and ATP hydrolysis coupled to proton transport. The results were similar to those of previous studies [11, 12]. Among the proteins encoded by housekeeping genes, such as ATP synthases, elongation factors, ribosomal proteins, or aminoacyl-tRNA synthetases, as well as proteins involved in carbohydrate metabolism [15, 19, 54], most species homologies were affiliated with *Sphingopaxix* or other genera of the Proteobacteria. Elongation factor Tu plays an important role in the elongation cycle of protein synthesis [10, 51]. Although the proteome analysis of community DZ21 is limited to species with high cell numbers or large amounts of biomass, this method generated valuable data that provided insight into *E. prolifera* degradation.

In summary, this is the first report of multiple "omics" analyses of marine macroalgae *E. prolifera* degradation. Microalgae and brown seaweed have been evaluated for biofuel production because of their higher photosynthetic efficiency, biomass yield, and growth rates. However, despite the high contents of carbohydrate and crude fiber in *E. prolifera*, its utilization as an energy source remains low worldwide. In this study, we conducted an in-depth analysis of the structure and function of and *E. prolifera*-degrading microbial community. Our results provide a foundation for determining the mechanism of functional genes for degradation and provide a theoretical foundation and technical support for constructing genetic engineering bacteria containing key enzymes. It can also suggest new methods of resolving marine ecological security issues caused by the marine macroalgae *E. prolifera*.

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