（様式５）(Style5)

学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

氏名：
Name Ratna Mondal

学位論文題目：
Title of Dissertation

Study on the enzymes involved in the later steps of degradation pathway of ulvan extracted from green algae *Ulva* sp.
（緑藻ウルバ属から抽出したウルバン分解経路における後期反応をつかさどる酵素についての研究）

学位論文要約：
Dissertation Summary

Insufficiency of the source of energy and environmental pollution hinders sustainable development worldwide. To overcome this problem, environmental pollution should be converted into renewable energy. The blooms of the green algae *Ulva* sp. are responsible for the coastal green tides. Accumulation of large amounts of algal biomass on beaches negatively affects human and animal health, tourism, aquacultures, and fisheries. For this reason, marine waste green algae need to be controlled by a chemical or enzymatic process. It is a lucrative idea of producing biofuels such as ethanol and n-butanol from green algal biomass due to not being competitive to agro foods for arable land, having fast growth rates, and absence of structural biopolymers such as lignin. In fact, it has been recently reported that green algae can be used for the production of ethanol.

Ulvan is a complex water-soluble sulfated polysaccharide in the cell wall of green algae belonging to the genus *Ulva*. It is composed of α-linked L-rhamnose-3-sulfate (Rha3S), β-linked glucuronic acid (GluA), α-linked iduronic acid (IduA), and β-linked D-xylose (Xyl). Ulvan polysaccharide and depolymerized oligo-ulvan can be used in various food, feed, biomedical, paint, and textile industries. Discovering new enzymes for the degradation of ulvan could improve the management and utilities of this resource. Ulvan-utilizing bacteria as a carbon source residing in the ocean are the promising sources for novel enzymes for the depolymerization and metabolization of algae-specific carbohydrates. Inspection of these ulvan-utilizing bacteria genomes reveals that the respective ulvan degrading genes are usually located in the gene clusters on the genome, which is called polysaccharide utilization locus (PUL). The involved carbohydrate-active enzymes (CAZymes) are listed and classified in the CAZy database (http://www.cazy.org/).

Ulvan lyase is the first enzyme for ulvan degradation, which catalyzes the cleavage of glycosidic bonds between Rha3S and uronic acids by elimination mechanism producing an unsaturated uronyl residue at the non-reducing ends of the formed products. Until now, three types of ulvan lyases, which belong to polysaccharide lyases (PLs) of families PL24, PL25, and PL28, have been identified. Polysaccharide lyase degradation products are oligosaccharides with an unsaturated uronyl residue at the non-reducing end. The predominant reaction mechanism for oligo saccharides degradation is hydrolysis, and more than 130 different glycosyl hydrolase (GH) families have been identified to date. In order to degrade ulvan completely to monosaccharides at the later steps, several enzymes further cleaving the unsaturated sugar are necessary. In this study, I have isolated several ulvan-utilizing bacterium and determined the draft genome sequences. I investigate the characteristics of the enzymes for the later steps of ulvan degradation inside cells.

Draft genome sequences of bacterial degrading strains of green algae *Ulva ohnoi* ulvan

In **Chapter 1**, I present the draft genome sequences of four marine bacterial strains, which can use ulvan as a sole carbon source extracted from green algae *Ulva ohnoi*. For this study, I isolated several ulvan utilizing bacteria which can use *U. ohnoi* as a sole carbon source from Uranouchi Bay, Kochi, Japan. Among these, I determined the draft genome sequences of four bacterial strains, KUL10, KUL17, KUL42, and KUL49. Based on partial nucleotide sequences of 16S rRNA genes, KUL10 was assigned as *Glaciecola sp.* and three other strains were classified as *Alteromonas sp*. The whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession numbers BGNG00000000, BJKJ00000000, BJKK00000000, and BJKL00000000 for KUL10, KUL17, KUL42, and KUL49, respectively. The versions described in this study are the first versions. The read archives have been deposited in DDBJ DRA.

Draft genome sequences of marine bacteria, which use *Ulva meridionalis* ulvan as a sole carbon source

In **chapter 2**, I present the draft genome sequences of bacterial strains belonging to genera *Alteromonas* and *Tenacibaculum*. For this study, *U. meridionalis* hung for one week in the Uranouchi bay (Kochi, Japan) was used as an enrichment source. Ulvan-utilizing bacteria were enriched in the artificial seawater with ulvan extracted

from *U. meridionalis* as a sole carbon source. In order to identify PULs and elucidate the evolution of ulvan degradation enzymes, I determined the draft genome sequences of seven bacterial strains, four *Alteromonas* sp. KUL106, KUL150, KUL154, and KUL156 and three *Tenacibaculum* sp. KUL113, KUL118, and KUL152. This whole-genome shotgun (WGS) project has been deposited at DDBJ/EMBL/GenBank under the accession numbers BLIG00000000, BLIH00000000, BLII00000000, BLIJ00000000, BLIK00000000, BLIL00000000, and BLIM00000000 for KUL106, KUL113, KUL118, KUL150, KUL152, KUL154 and KUL156 respectively. The versions described in this study are the first versions. The read archives have been deposited in DDBJ DRA.

An ulvan-utilizing *Alteromonas* differentiates two PL24 ulvan lyases for ulvan degradation

In **chapter 3**, I studied to elucidate how ulvan lyases work in the ulvan degradation pathway and to discover more ulvan-degrading bacteria, which can produce novel ulvan-degrading enzymes and contribute to potential use of ulvan as oligosaccharides. For this study, the genome of an ulvan-utilizing bacteria *Alteromonas* KUL106 strain was curated and two long-type PL24 ullA genes, three short-type PL24 ullB genes, and one PL25 ullC gene were found. The long-type ulvan lyase is composed of two modules, catalytic module, which is similar to short-type ulvan lyase, and the C-terminal module. Truncated *ullA* deleting C-terminal half, three *ullB* genes and *ullC* were cloned in pET22b with His-tag on the C-terminal end and heterologously expressed in *Escherichia coli* BL21(DE3). Overexpressed proteins were purified with a nickel column to homogeneity. All the purified proteins showed ulvan lyase activities, although the activities of UllB2 and UllB3 were weak because no reaction products were observed in the reaction mixture of UllB2 and UllB3 after 1 h incubation. Kinetic parameters for ulvan and oligo-ulvans were measured with UllA, UllB, and UllC. UllA reacted much more with ulvan than oligo-ulvans. C-terminal truncated long-type Pl24 ulvan lyase UllA work outside cells to degrade ulvan into oligo-ulvans. This study using the KUL106 strain supports the data. Since the long-type ulvan lyase showed a very weak affinity to the oligo-ulvan, it does not react with oligo-ulvans inside cells. UllB reacted with ulvan and oligo-ulvans in a similar way. Instead, UllB prefers oligo-ulvans. Actually, UllB has no signal sequence and does not seem to be secreted outside cells. UllC reacted with oligo-ulvans higher than ulvan. The affinities of all enzymes were higher to ulvan than oligo-ulvans.

To know the localization of lyases, secreted proteins from KUL106 and soluble proteins in KUL106 were partially separated using a Q Sepharose anion-exchange chromatography, and proteins with ulvan lyase activity were detected. The long-type PL24 ulvan lyase UllA was detected only in the secreted proteins and the short-type UllB was detected only in the soluble fraction. Altogether, I concluded that while secreted UllA mainly reacted with ulvan, UllB was a major ulvan lyase reacted with oligo-ulvans inside cells. Since UllC was not detected from KUL106 cells, the function of UllC is still unclear.

Characterization of *Glaciecola* sp. enzymes involved in the late steps of degradation of sulfated polysaccharide ulvan extracted from *Ulva ohnoi*

Until now, several ulvan lyases have been discovered which degradation products are oligosaccharides with a degree of polymerization higher than two. Clear and complete degradation of ulvan is still unknown. For the complete degradation of the products of polysaccharides lyase, microbial genomes simultaneously encode enzymes that can further cleave the unsaturated sugar. To acquire this goal, in **Chapter 4**, I have identified an ulvan associated polysaccharide utilization locus (PUL) residing between two ulvan lyase genes belonging to families of polysaccharide lyase 24 (PL24) and PL25 in the genome of a ulvan-utilizing bacterium *Glaciecola* KUL10 strain. The PUL contains many genes responsible for oligo-ulvan degradation. I choose two GH105 genes, KUL10_26540 and KUL10_26770 and one GH88 gene KUL10_22590 for the possible β-glucuronyl hydrolases and four genes, KUL10_26590, KUL10_26600. KUL10_26760, and KUL10_26780, for rhamnosidases and cloned into the expression vector pET21a, gene products were overexpressed in *E.coli* BL21 (DE3) and purified. Among them, I found that both KUL10_26540 and KUL10_26770 had an unsaturated β-glucuronyl hydrolase activity to produce Rha3S and oligosaccharides, such as Rha3S-GluA-Rha3S, Rha3S-IduA-Rha3S and, Rha3S-Xyl-Rha3S, by releasing 5-dehydro-4-deoxy-D-glucuronate. I analyzed the unsaturated disaccharide and tetrasaccharide and their reaction products with KUL10_26540 using the Aminex HPX-87H organic analysis column. At retention time 11.2 min, the specific peaks were observed only in the products from both unsaturated disaccharide and tetrasaccharide. This could be 5-dehydro-4-deoxy-D-glucuronate. However, KUL10_26540 showed much higher activity than KUL10_26770

and was more active on disaccharide than tetrasaccharide.

I also found rhamnosidase activity for four KUL10 gene products KUL10_26600, KUL10_26780, KUL10_26590, and KUL10_26760 against the synthetic substrate PNP-α-L-rhamnopyranoside, although they could not react on the sulfated rhamnose. The desulfated Rha-Xyl-Rha3S is cleaved to Xyl-Rha3S by the CBM67 family enzyme of an ulvan-degrader *Formosa agariphila*. These suggest that the rhamnosidase is active only on desulfated rhamnose.