Biochemical characteristics of cellulose and a green alga degradation by *Gilvimarinus japonicas* 12-2\(^T\), and its application potential for seaweed saccharification

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**ABSTRACT**

Cellulose is one of the major constituents of seaweeds, but reports of mechanisms in microbial seaweed degradation in marine environment are limited, in contrast to the multitude of reports for lignocellulose degradation in terrestrial environment. We studied the biochemical characteristics for marine cellulolytic bacterium *Gilvimarinus japonicas* 12-2\(^T\) in seaweed degradation. The bacterial strain was found to degrade green and red algae, but not brown algae. It was shown that the bacterial strain employs various polysaccharide hydrolyses (endocellulase, agarase, carrageenanase, xylanase, and laminarinase) to degrade seaweed polysaccharides. Electrophoretic analysis and peptide sequencing showed that the major protein bands on the electrophoresis gel were homologous to known glucanases and glycoside hydrolases. A seaweed hydrolysate harvested from the bacterial culture was found useful as a substrate for yeasts to produce ethanol. These findings will provide insights into possible seaweed decomposition mechanisms of *Gilvimarinus*, and its biotechnological potential for ethanol production from inedible seaweeds.

Cellulose is one of the most abundant components of biomass in terrestrial environments, and is a prevalent marine polysaccharide, which acts as a structural glucan in seaweeds [1]. However, reports of the isolation of marine cellulolytic microbes are limited, in contrast to the multitude of reports for soil microbes. Amongst marine microbes, *Saccharophagus* sp. strains are intensively studied as a model for seaweed decomposition [2–6]. Culture-independent metagenome studies suggest that many microbes in natural habitats have not been cultivated [7,8]; so, many cellulolytic microbes involved in seaweed degradation remain to be isolated. To fully elucidate their role in the seaweed decomposition, it is necessary to isolate cellulolytic microbes from marine environments that have not been cultured. In addition, an exploration of the novel strains and the potential use of their enzymes in industrial applications is valuable from a biotechnological viewpoint, because seaweed hydrolysate is in demand in the food, textile, animal husbandry, and biofuel industries [9].

We have recently isolated cellulolytic marine bacterial strain 12-2\(^T\) from a piece of cotton rope fragment washed ashore on a beach, and found that the strain is a novel species of the genus *Gilvimarinus*, designated as *G. japonicas* [10]. *Gilvimarinus* was established as a new genus in 2009, and only three species, including our strain, were accepted by the International Committee on Systematics of Prokaryotes [11,12]. Since all *Gilvimarinus* members are reported to possess agarolytic activity, they appear to contribute to seaweed decomposition in marine environment, but many characteristics, including ecological role and a mode of enzyme action for seaweed degradation are still unclear.

In the present study, we examined the microbial degradation activity of several typical seaweeds by *G. japonicas* 12-2\(^T\), and analyzed the enzymatic profile of the bacterial strain for polysaccharide hydrolysis, to understand the ecological role of *Gilvimarinus* in seaweed biodgradation and its potential biotechnological applications.

**Materials and methods**

**Chemicals and materials**

Polysaccharides, including crystalline cellulose (Avicel), carboxymethyl cellulose, laminarin, xylose, mannann, sodium alginate, and carrageenan, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Microbiology media, including marine broth, yeast extract, malt extract, and peptone, were obtained from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Marine Art SF-1, chemically defined artificial seawater, was purchased from Tomita Pharmaceuticals (Tokushima, Japan). FG-DMP7 dialysis tubes (MWCO
1,000) and VIVASPIN-20 ultrafiltration spin columns (MWCO 10,000) were purchased from Nippon Genetics (Tokyo, Japan), and Sartorius (Goettingen, Germany), respectively. EzStain Reverse kit, for reverse staining of protein was obtained from ATTO (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemicals (Kyoto, Japan).

Yeast strains (Saccharomyces cerevisiae NBRC-104019T, Lachancea cibidi NBRC16841T, Pichia heimii NBRC16865T, Debaryomyces yamadae NBRC10691T, and Zygosaccharomyces rouxii NBRC11301T), for fermentation studies, were obtained from NITE Biological Resource Center (Kazusa, Japan).

**Collection of seaweeds**

Seaweeds, including a green alga (Monostroma nitzidum), a red alga (Calliblepharis saidana), a brown alga (Sargassum confusum), and an eelgrass (Zostera marina) were collected from a seashore of the Aio district in Yamaguchi city in April, 2016. The seaweeds were washed in 10 L of deionized water three times to remove salts and freeze-dried using a FD1000 Freeze drier (Eyela, Tokyo, Japan).

**Seaweed degradation activity of G. japonicus 12-2T**

A single colony of G. japonicus 12-2T on marine agar slant was used to inoculate 10 mL marine broth and cultivated at 150 rpm, 25°C for 1 week to use as a seed culture. An aliquot (0.1 mL) of the seed culture was added to 20 mL of seaweed artificial seawater (SAS) medium composed of 38.2 g L⁻¹ marine Art SF-1, 20 g L⁻¹ of dried seaweeds (M. nitzidum, C. saidana, S. confusum, or Z. marina), 5.0 g L⁻¹ peptone, and 1.0 g L⁻¹ yeast extract, and cultured at 150 rpm, 25°C for 15 days. During the culture, aliquots of the culture were collected every 3 days, centrifuged (20,000 × g, 20 min, 4°C) to harvest the supernatant, and analyzed for reducing sugar yield (Miller, 1959; Wood and Bhat, 1988).

**Preparation of hydrolase cocktail**

A single colony of G. japonicus 12-2T was used to inoculate 10 mL marine broth and cultivated at 150 rpm, 25°C for 1 week to use as a seed culture. The seed culture was added to 2 L of filter paper artificial seawater (FPAS) medium (38.2 g L⁻¹ Marine Art SF-1, 5.0 g L⁻¹ peptone, 1.0 g L⁻¹ yeast extract, and 20 g L⁻¹ Whatman no.7 filter papers (3 × 20 mm fragments cut by a paper shredder)) and SAS medium (described above with green alga (M. nitzidum) as a substrate), and cultivated at 150 rpm, 25°C for 2 weeks to produce the enzyme mixture involved in polysaccharide degradation (hydrolase cocktail).

After a 2-week cultivation, cultures were filtered through a threefold gauze to remove filter paper or seaweed residues. The filtrate was subsequently centrifuged (10,000 × g, 20 min, 4°C) to separate the supernatant containing enzymes secreted by the bacterial cells. Ammonium sulfate was dissolved in the resultant supernatant to attain a final concentration of 80% saturation, after which the protein precipitate was obtained by centrifugation (20,000 × g, 30 min, 4°C). The precipitate was dissolved in 20 mL of artificial seawater (38.2 g L⁻¹ Marine Art SF-1), dialyzed using a DM-P7 dialysis tube to remove ammonium sulfate, and concentrated to 10 mL, using a VIVASPIN-20 ultrafiltration column. Bacterial cells were washed with 10 mL of artificial seawater three times and suspended into 10 mL of artificial seawater containing 0.1% (w/V) sodium azide to prepare a cell suspension.

**Biochemical analysis of the hydrolase cocktail**

Exocellulase, endocellulase, agarase, laminarinase, alginate lyase, mannanase, carrageenanase, and xylanase activity were determined using Avicel (microcrystalline cellulose) [13], carboxymethyl cellulose (amorphous cellulose) [14], agarose, laminarin, sodium alginate, mannan, carrageenan, and birchwood xylan as the substrates, respectively, with the enzyme reaction carried out at 37°C for 4 h [15]. The hydrolase activities were determined by quantifying reducing sugar formation using the 3,5-dinitrosalicylic acid (DNS) method at 540 nm [16]. The units of hydrolase activity were defined as the amount of enzyme required to release 1 μmol of reducing sugar per min under the reaction conditions. Glucose was used as a standard for quantifying reducing sugars from cellulose, laminarin, and alginate, while galactose was used for quantifying reducing sugars from agarose and carrageenan. Xylose and mannose were used for quantifying reducing sugars from xylan and mannan, respectively. The concentrated culture supernatant and cell suspension were used to examine the activity of secreted hydrolases and cell-associated hydrolases, respectively.

The concentrated culture supernatant was subjected to SDS-PAGE in 6, 8, and 12% gels on an NA-1012 electrophoresis module (Nippon Eido, Tokyo, Japan) to separate the secreted enzymes, which were then visualized by reverse staining. Additionally, each protein band in the SDS-PAGE gel was blotted on a PVDF membrane and stained with a Coomassie Brilliant Blue R-250, and their N-terminal 10 amino acid residues were analyzed by a PPSQ21A protein sequencer (Shimadzu, Kyoto, Japan). These amino acid sequences were
subjected to the Protein BLAST algorithm to analyze candidate proteins in known glucanase and gluco-side hydrolase families [17].

**Bioethanol fermentation from seaweeds**

A single colony of *G. japonicus* 12-2<sup>T</sup> was inoculated in 10 mL of marine broth and cultured at 150 rpm, 25°C for 1 week to use as a seed culture. An aliquot (0.1 mL) of the seed culture was added to 20 mL of SAS medium supplemented with a green alga (*M. nitidum*) and cultivated at 150 rpm, 25°C for 1 week. Supernatant culture was recovered by a centrifugation (10,000 × g, 20 min, 4°C) and used as a seaweed hydrolysate. Ten milliliters of the hydrolysate was mixed with 50 mg peptone, 10 mg yeast extract, and 500 μg chloramphenicol in a glass vial. Subsequently, a single colony of yeast was inoculated to the mixture, followed by a nitrogen flush of the gas phase in the vial for 1 min, and sealing with butyl-rubber and aluminum caps. Fermentation was carried out for 1 week at 37°C, and ethanol yield in the fermentation broth was determined using a F-kit ethanol diagnostic reagent (Roche Diagnostics, Basel, Switzerland), according to manufacturer’s instructions.

**Results and discussion**

**Degradation activity of *G. japonicus* 12-2<sup>T</sup> on common seaweeds**

We determined the type of seaweed (green alga, red alga, brown alga, and eelgrass) that is a substrate for *G. japonicus* 12-2<sup>T</sup> in its natural habitat by evaluating the yield of reducing sugars during culture, on feeding with each seaweed as a carbon source, and the result is shown in Figure 1. Reducing sugar was found to be increased in the bacterial culture fed with green alga and red alga, while neither bacterial growth nor sugar production was observed in the cultures fed with brown alga and eelgrass. Therefore, it is suggested that the bacterial strain is mainly involved in biodegradation of green algae and red algae, but not that of brown algae and eelgrass, and the variation in biodegradability of seaweeds is due to the differences in polysaccharide composition. In contrast, biodegradation resistance of eelgrass is likely due to the fact that it contains lignin as well as cellulose [18], because phenolic parts in lignin is known to inhibit cellulase activity [19].

**Enzyme profile for the hydrolase cocktail**

The major polysaccharide composition in each type of seaweed is unique. For instance, green algae contains cellulose, xylan, and mannan; red algae contains agarose, carrageenan, and cellulose; and brown algae contains alginate and cellulose as structural glucans, and laminarin as a storage glucan [1]. In our previous work, we confirmed that *G. japonicus* 12-2<sup>T</sup> could grow with filter paper or agarose as a carbon source [10], but the enzyme activity for polysaccharide hydrolases remains unknown. As described above, the strain 12-2<sup>T</sup> showed the highest saccharification activity to a green alga among tested seaweeds (Figure 1). Therefore, the enzyme activity profile for secreted hydrolases of the strain 12-2<sup>T</sup> fed with filter paper and green alga (*M. nitidum*) was assessed. Supernatants of the bacterial culture fed with filter paper and culture fed with green alga were harvested, and their hydrolase activities were determined (Figure 2a,b). Endocellulase and xylanase activity were found in all the culture supernatants, though exocelullase was not detected, suggesting that *G. japonicus* 12-2<sup>T</sup> mainly degrades amorphous parts of the seaweed cellulose. Xylan is mainly found in green algae [20,21], indicating that the bacterial strain also participates in xylan decomposition in the seaweed. The reason for production of xylanase in the filter paper-fed culture may be the induction of xylanase gene expression by cellulose or its degradation metabolites such as glucose or cellobiose, which have been reported to act as inducers in both soil and marine microbes [4,6,22,23]. Weak agarase activity was found in the seaweed-fed culture, but not in the filter paper-fed one, suggesting that agarase gene expression is induced by some seaweed constituents, but not by cellulose or its metabolites. Interestingly, laminarinase activity was also observed in filter paper-fed culture, but alginate lyase activity was not observed in any culture. *Gilvimarinus japonicus* 12-2<sup>T</sup> could not degrade the brown alga, as mentioned above, and laminarin is a polysaccharide formed from glucose. Therefore, it is suggested that *G. japonicus* 12-2<sup>T</sup> can assimilate the storage glucan (laminarin), but not the
structural glucan (alginate) in brown algae, and that the bacterial strain seems to participate in the late stage of brown algae decomposition in which alginate would be degraded by other microbes and laminarin becomes exposed. While mannan is one of the polysaccharides found in green and red algae [21,24], mannanase activity was not detected in any culture. Subsequently, enzyme activity of the cell-associated hydrolases was examined (Figure 2c,d). Endocellulase and xylanase activity was found in the cell suspensions from filter paper-fed and seaweed-fed culture, which was similar to the enzyme profile observed for the supernatant. Evaluation of enzyme activity in the cell suspension implied that *G. japonicus* 12-2T possesses cell-associated and secreted forms of endocellulase and xylanase. The presence of these enzymes is likely because several bacterial strains are reported to possess cell-associated cellulase [25–28]. Carrageenanase activity was found exclusively in cell suspension, suggesting that the strain possesses only cell-associated carrageenanase. Although many carrageenanase-producing bacteria are known, most of them produce the secreted form of carrageenanase [29]. Recently, *Zobellia galactanivorans*, a marine Bacteroidetes, was found to possess carrageenanase on its outer membrane, which is the first reported for cell-associated carrageenanase [30]. To the best of our knowledge, there is no report for cell-associated carrageenanase for Proteobacteria, thus our data provide the first report for this phylum.

Recently, the whole genome of *G. agarlyticus* was sequenced, and many polysaccharide hydrolase genes including cellulase, agarase, xylanase, and other glucanases were identified [31]. The genome sequence for *G. polysaccharolyticus* and *G. chinensis*, which was published in the Genbank database under accession nos. LFJ0000000.1 and ARIX0000000.1, respectively, also reported those enzyme genes. Therefore, cellulolytic and agarolytic activity appears common characteristics for the *G. japonicus* members. In contrast, genes for mannanase and alginate lyase, whose enzyme activities were not observed in our assay conditions, were also found in the whole genome sequence of known neighbors, implying that *G. japonicus* 12-2T possesses those enzyme genes but does not express them under the culture condition employed in this study. Optimum culture condition remains to be established for mannanase and alginate lyase analysis.

**Determination of the n-terminal amino acid sequences for secreted hydrolase enzymes**

Each hydrolase enzyme secreted in the filter paper-fed culture of *G. japonicus* 12-2T was separated by SDS-PAGE and blotted on a PVDF membrane to determine the N-terminal amino acid sequences for enzymes in the hydrolase cocktail. Figure 3 shows the electrophoresis gel image for the hydrolase cocktail obtained from the glucose-fed, filter paper-fed, and seaweed (green alga)-fed
and analyzed using a Protein BLAST search, and the close known proteins hit by the search are shown in Table 1 and S1. While Table 1 shows a list of the known proteins which are the closest to major proteins found in the strain 12-2T culture, Table S1 includes an entire list of all the candidates for them. Most protein bands were found similar to polysaccharide hydrolases for marine bacteria as well as those for some soil bacteria, suggesting that further study of enzymology is necessary to understand bacterial metabolisms in marine seaweed decomposition. Considering the hydrolase varieties found together in the enzyme assay, G. japonicus 12-2T appears to employ several hydrolase enzymes to assimilate green and red algae efficiently as its carbon source. We also attempted to analyze the separation of enzymes secreted in the seaweed fed-culture, but a dense smear, likely derived from seaweed polysaccharides and proteins, inhibited the separation and visualization of the 12-2T enzyme bands (Figure 3).

### Bioethanol fermentation from seaweed

The availability of the seaweed (green alga *M. nitidum*) hydrolysate produced by *G. japonicus* 12-2T for ethanol production was assessed. Since the hydrolysate is thought to contain salts derived from artificial seawater, we prepared cultures of osmotic tolerant yeast strains (*L. cidri*, *P. heinitii*, *D. yamadae*, and *Z. rouxii*), as well as, of conventional yeast *S. cerevisiae*. Figure 4 shows the ethanol yields from the seaweed hydrolysate by yeast strains after 1 week of fermentation. Glucose-fed *S. cerevisiae* produced 60 mg ethanol per g-glucose by 1-week fermentation, and seaweed hydrolysate-fed *S. cerevisiae* and osmotic tolerant yeast strains produced approximately 40 mg ethanol per g-dried seaweed. Ethanol yield obtained in our assay was comparable to ones from enzymatically saccharified seaweed reported in published

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**Table 1.** The closest amino acid sequences of known proteins for major proteins in *G. japonicas* 12-2T culture supernatant using the Protein BLAST algorithm.

<table>
<thead>
<tr>
<th>Band</th>
<th>Queried sequence</th>
<th>Protein name</th>
<th>Protein ID</th>
<th>Subjected sequence</th>
<th>Identity (%)</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>GPEAHPTWW</td>
<td>Endoxylanase [Aeromonas caviae]</td>
<td>BA133641.1</td>
<td>164 PDYAHPTWW 177</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>B</td>
<td>WPPAGDYNWE</td>
<td>1,4-β-xylanase [Oceanobacillus sp. Castelsara]</td>
<td>WP_002918135.1</td>
<td>292 WPPAGDYNWE 102</td>
<td>70</td>
<td>90</td>
</tr>
<tr>
<td>C</td>
<td>GQVEGVGFSE</td>
<td>Glycoside hydrolase [Marinimicrobia bacterium 46, 47]</td>
<td>KUK56294.1</td>
<td>492 GQVEGFSE MFSE</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>E</td>
<td>GQQAQWAEEQ</td>
<td>α/β-hydrolase [Serratia marcescens]</td>
<td>WP_000430255.1</td>
<td>232 WQQAQWAEEQ 231</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td>F</td>
<td>AHWPNWVWWV</td>
<td>α/β-hydrolase [Burkholderia sp. AU6039]</td>
<td>WP_085497406.1</td>
<td>535 AHWPNWVWWV</td>
<td>70</td>
<td>80</td>
</tr>
<tr>
<td>G</td>
<td>ALEQTQWWNA</td>
<td>Glycoside hydrolase [Paenibacillus sp. SS-1]</td>
<td>OXLE7686.1</td>
<td>210 ALEQTQWWNA</td>
<td>70</td>
<td>80</td>
</tr>
<tr>
<td>H</td>
<td>APKKWKKKWW</td>
<td>Glycoside hydrolase-16 protein [Millonella massiliensis]</td>
<td>WP_071135321.3</td>
<td>31 APKKWKKKWW</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>I</td>
<td>WGGTAEWWVWV</td>
<td>α-mannosidase [Butyribrio fibrisolvens]</td>
<td>WP_027215840.1</td>
<td>102 WGGTAEWWVW 111</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>J</td>
<td>AQEGQQWWWWH</td>
<td>Glycoside hydrolase [Paenibacillaceicola gangotriensis]</td>
<td>WP_0072771102.1</td>
<td>11 VREEQWWWWQ</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>K</td>
<td>AQPQTGATG</td>
<td>Endoglucanase [Cellulosilyticum ruminicola]</td>
<td>ACZ98593.1</td>
<td>170 AQPQTGATG</td>
<td>70</td>
<td>90</td>
</tr>
<tr>
<td>L</td>
<td>AQQTAGTG</td>
<td>Endoglucanase [Cellulomonas cellulosa]</td>
<td>KGM02499.1</td>
<td>627 AQQTAGTG</td>
<td>70</td>
<td>90</td>
</tr>
</tbody>
</table>
studies [32], and thus a reaction process involving both physicochemical pretreatment and enzymatic hydrolysis might improve ethanol production. The bioconversion of inedible seaweeds grown in eutrophic sea areas to valuable chemicals such as bioethanol is regarded as an attractive way to utilize the marine waste biomass. While it is worth noting that the seaweed hydrolysate produced by G. japonicus 12-2T culture can be a substrate for conventional yeast, there is room to improve ethanol yield. One way to improve yield would be to establish optimal saccharification and fermentation conditions. Additionally, employment of nonconventional yeasts, which can assimilate sugars such as cellobiose, galactose, or xylose, generated by saccharification of cellulose, agarose, or xylan, respectively, is a promising avenue to pursue, which is in progress and the results will be reported in the near future.

Conclusion

In this study, we examined the biochemical characteristics of the marine cellulolytic and agarolytic bacterium G. japonicus 12-2T during seaweed degradation. A series of experiments examining seaweed degradation and hydrolysis activity suggest that G. japonicus 12-2T is involved in the decomposition of green and red algae by using several secreted and cell-associated forms of polysaccharide hydrolases. Additionally, the results indicate that the bacterial strain contributes to brown algae degradation in the late stage of decomposition by assimilating laminarin. Recently, a marine bacterial strain, Saccharophagus sp., caught the attention of marine microbiologists due to their potent seaweed degradation activity. The genus Gilvimarinus seems to participate in seaweed decomposition in the same manner as other marine seaweed degraders such as Saccharophagus, which contributes to carbon cycle in the marine environment. In addition, the polysaccharide hydrolases are essential for developing seaweed hydrolysate, a useful substrate for several industries. Further studies regarding the functions of these enzymes in Gilvimarinus are necessary to understand the microbial ecology involved in seaweed degradation.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contribution

K. F. designed the experiments. S. K. mainly performed the experiments and wrote the manuscript under supervision of K. F.

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