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Continuous Saccharification of Laminarin by Immobilized Laminarinase ULam111 Followed by Ethanol Fermentation with a Marine-Derived Yeast

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Abstract

We isolated a novel laminarinase ULam111 from *Flavobacterium* sp. strain UMI-01. Purified ULam111 showed degradation activity against laminarin with the specific activity of 224 ± 18 U/mg at 30˚C and pH 6.0. Its optimum temperature was 50˚C, and degradation activities against laminarin were observed at 4˚C - 80˚C. With a laminarin degradation system, we investigated the preparation and properties of immobilized ULam111 with the use of the 11 types of carriers. The high activity recoveries of immobilized ULam111 were as follows: 19.4% for IB-S60P carrier beads (the non-ionic type), 15.6% for IB-S60S carrier beads (the non-ionic type), 11.9% for IB-150P carrier beads (the covalent type), and 7.1% for IB-C435 carrier beads (the cationic type). With the repeated use of immobilized ULam111, the enzyme activities immobilized on IB-S60S and those on IB-S60P remained at 40% and 30% respectively after the sixth trial. We selected IB-S60S as suitable beads for enzyme immobilization, and we attempted to construct a reactor system with ULam111 immobilized on IB-S60S beads. In this system, 1.2 - 1.9 g/L glucose was repeatedly produced from 30 mg/mL laminarin solutions after 20 hr when the reactor operation was repeated 10 times. We examined ethanol fermentation from the saccharified solutions with a marine-derived yeast (*Saccharomyces cerevisiae* C-19), and 0.51 - 0.58 g/L bioethanol was produced from the saccharified solution that contained 1.71 - 1.86 g/L of glucose.

Keywords

Laminarin, Laminarinase, β-1,3-Glucanase, Immobilization, Ethanol Fermentation

1. Introduction

Seaweeds account for the majority of the worldwide mass of marine organisms. Of these, brown algae produce unique and edible polysaccharides such as laminarin, alginate, and fucoidan. Laminarin is a storage polysaccharide that belongs to the family of β-glucans, and its main chain consists of glucose through β-1,3 glycosidic bonds. In addition, chains via β-1,6 glycosidic bonds partially branch [1] [2]. Laminarin is classified into two types based on their terminal structures. Accordingly, the M-chain and the G-chain have D-mannitol and glucose at reducing end, respectively [3]. The content of laminarin in brown algae is up to 32%–35% of dry weight in *Laminaria digitata*, *Laminaria hyperborea*, and *Saccharina latissima* and these values change seasonally [4]. To date, laminarinases are found in some species of bacteria, fungi, yeasts, and marine invertebrates [5]. The ratio between the β-1,3 and β-1,6 bonds in laminarin was 7:1 in *L. digitata* [5] and 3:2 in *Eisenia bicyclis* [6], and β-1,3-glucanase have a primary role in the effective degradation of laminarin. According to the CAZy database [7], β-1,3-glucanases are classified into glycosidehydrolase families (GH) 16, 17, 55, 64, 81, and 128. Of these, β-1,3-glucanases belonging to the GH16 family are abundant and well characterized. Labourel *et al.* [8] reported the characterization of GH16 laminarinase from *Zobellia galactanivorans* and solved its structure, suggesting that a bacterium associated with brown algae in nature uses glucose derived from brown algal laminarin.

Seaweeds are thought to be promising biomass feedstock for bioethanol production because they contain no lignin; thus, a simple biorefinery process can be used to produce sugars from seaweed. For example, polysaccharides can be easily decomposed from the seaweed to sugars with enzymatic treatment [9] [10]. As high ethanol-fermenting yeasts (e.g., *Saccharomyces cerevisiae*) cannot degrade polysaccharides, it is important to degrade the polysaccharides to mono- or oligo-saccharides. Thus, for the utilization of the wastes of processed brown alga as a raw material for the production of bioethanol, the necessary the first step is a saccharification process, *i.e.*, an enzymatic degradation of polysaccharides such as laminarin. As the second step, glucose produced by a laminarinase is fermented with the yeast. Notably, Motone *et al.* created a yeast with laminarinase by cell surface engineering [11]. Hydrolysis with an enzyme is often applied for the construction of a multiple parallel fermentation system for the brewing of sake, but there are few reports about the application of enzyme immobilization in such a system.

Methods of enzyme immobilization can be divided into carrier-bound technology, which is anchoring between a solid support (the carrier) and the enzyme, and other methods; e.g., entrapment, and cross-linking [12] [13] [14]. Solid support can be divided into organic polymers and inorganic polymers (e.g., silica, glass). Organic polymers can be sub-divided into natural polymers (e.g., cellulose, alginate, collagen, carbon) and synthetic polymers (e.g., polystyrene, polyacrylic, polypropylene) [12] [13] [14]. The carriers used for enzyme immobilization have one or more kinds of functional group (e.g., epoxy group,

amino group, carboxylic acid), and are linked enzymatic protein by its functional group [12] [13]. Many types of immobilization beads are currently available on the market [13]. Various types of enzymes such as lipase, penicillin acylase, laccase and glucose isomerase have been applied in many industries with the use of immobilization [15] [16] [17]. There are also several reports on the immobilization of polysaccharide-degrading enzymes such as alpha-amylase [18], cellulose [19], and laminarinase [20]. From a cost-effectiveness viewpoint, both alpha-amylase and cellulose are cheap and there is little need for their repeated use. In contrast, the cost of laminarinase is very high and its immobilization is thought to be a useful method.

In the present study, a novel β-1,3-glucanase from *Flavobacterium* sp. strain UMI-01, which had been isolated from decayed brown algae [21], was enzymatically characterized with laminarin as the substrate and we named it ULam111. We then assessed the activity and operational stability of the immobilized laminarinase ULam111 using many types of carrier. We also carried out a continuous saccharification of laminarin using immobilized ULam111 and examined ethanol fermentation from the saccharified solution with the marine-derived yeast.

2. Materials and Methods

2.1. Materials

L. digitata laminarin was purchased from Sigma-Aldrich (St. Louis, MO, USA), and *E. bicyclis* laminarin was from Tokyo Chemical Industry (Tokyo). We purchased the laminari-oligosaccharides: laminaribiose (L2), laminaritriose (L3), laminaritetraose (L4), laminaripentose (L5) and laminarihexsose (L6) from Megazyme (Bray, Ireland). The immobead enzyme carrier kit was purchased from ChiralVision (Leiden, The Netherlands).

2.2. Construction of Recombinant ULam111 Expression System

Genomic DNA from *Flavobacterium* sp. strain UMI-01 was prepared using ISOHAIR (Nippon Gene, Tokyo) as described [22]. A DNA encoding ULam111 (GenBank accession no. LC202090) was amplified by genomic polymerase chain reaction (PCR) using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) with a primer set of F1 (5'-AGGTAATACACCATGACTAAAGGAAAAAAACTCGGT-3') and R1 (5'-CACCTCCACCGGATCCTTGATACACCTTAATATAGTC-3'). The thermal cycling conditions were: initial denaturation at 98˚C for 30 sec, 30 cycles of denaturation at 98˚C for 10 sec, annealing at 55˚C for 15 sec, and extension at 72˚C for 45 sec. Amplified DNA was ligated into a modified pCold vector (Takara, Shiga, Japan), which has been reported [23], using the In-Fusion HD Cloning Kit (Takara). After a sequencing analysis, recombinant plasmid DNA was introduced to *E. coli* BL21 (DE3) (Nippon Gene) or *E. coli* Rosetta-gami™ 2 cells (Novagen, Darmstadt, Germany).

2.3. Expression and Purification of ULam111

(i) For the determination of the enzymatic properties of ULam111

E. coli BL21 (DE3) transformants were cultured at 37˚C for 16 hr in LB medium supplemented with 50 μg/mL ampicillin. After incubation at 15˚C for 1 hr, isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added for a final concentration of 0.1 mM, and the medium was cultured at 15˚C for 12 hr. All subsequent steps were carried out at 4˚C unless otherwise noted. Cells were harvested by centrifugation at 5000 g for 15 min and sonicated with a buffer containing 10 mM sodium phosphate buffer (pH 8.0), 0.5 M NaCl, 1% Triton X-100, and 0.01 mg/mL lysozyme. Supernatants after centrifugation at 10,000 g for 15 min were mixed with 200 μL of TALON Cobalt Resins (Takara) and incubated for 30 min. Resins were collected by centrifugation at 1500 g for 5 min and were washed with a buffer containing 20 mM imidazole-HCl (pH 8.0) and NaCl. Protein elution was conducted with a buffer containing 150 mM imidazole-HCl (pH 8.0) and NaCl. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), fractions containing ULam111 were dialyzed against 10 mM sodium phosphate buffer (pH 6.0) and 100 mM NaCl. The presence of the purified protein was confirmed by 10% (v/v) SDS-PAGE [24] with Coomassie Brilliant Blue R-250 dye. The protein concentration was determined by the Lowry method [25] using a DC Protein assay kit with bovine serum albumin (BSA) (Bio-Rad, Carlsbad, CA) as the standard.

(ii) For the immobilization of ULam111

For the overexpression of ULam111, we grew *E. coli* Rosetta-gami™ 2 transformants in LB medium containing 50 μg/mL ampicillin at 37˚C. The expression of the ULam111 gene was induced by cold shock with shaking in water and ice until the temperature of a culture solution decreased to below 15˚C and the addition of 1 mM IPTG to the medium. After a further incubation at 15˚C overnight, the cell pellet was collected by centrifugation, suspended in 50 mM potassium phosphate buffer (pH 7.5), disrupted by sonication, and centrifuged twice at 20,000 g for 30 min at 4˚C. The resulting supernatant was applied to a Ni Sepharose™ affinity column (GE Healthcare, Buckinghamshire, UK) (30 × 10 mm) and eluted in a stepwise manner with 8 mL of 25 mM, 75 mM and 200 mM imidazole in 50 mM potassium phosphate buffer (pH 7.5) and 0.5 M NaCl. The fractions containing ULam111 were concentrated using an Amicon Ultra device (Millipore, Bedford, MA) and dialyzed in 10 mM sodium phosphate buffer (pH 6.0), and ULam111 was thus partially purified.

2.4. Enzyme Immobilization for Bead Screening

For the screening of immobilization beads, we mixed 200 μL of ULam111 (1.12 unit) with 50 mg of each type of beads in 10 mM sodium phosphate buffer (pH 6.0) and incubated the mixture at 4˚C overnight. After the incubation, the supernatant was removed and unbound protein was washed away three times with 0.5 mL of the same buffer.

2.5. The Measurement of β-1,3-Glucanase Activity for the Determination of the Enzymatic Properties of ULam111

The enzyme reaction was carried out in a solution containing 10 mM sodium phosphate buffer (pH 6.0), 0.1 M NaCl, 0.1 mg/mL BSA, 2.0 mg/mL laminarin from *L. digitata*, and 0.001 mg/mL ULam111 at 30˚C for 15 min unless otherwise stated. The degradation activity was determined by measuring the liberated sugar by the method of Prado *et al.* (1998) [26]. One unit was defined as the amount of the enzyme that releases 1 μmol of glucose per min.

2.6. The Laminarin-Degradingenzyme Assay for Immobilized ULam111

Unless otherwise stated, the standard assay was carried out at 40˚C, using 10 mL of 1.5 mg/mL laminarin from *E. bicyclis* in 10 mM sodium phosphate buffer (pH 6.0). After the reaction was stopped by heat treatment at 100˚C for 10 min, we determined the amount of reducing sugars by absorbance at 660 nm using the method of Somogyi-Nelson [27] [28] with glucose as a standard. One unit of activity is defined as the amount of enzyme required to release 1 μmol glucose equivalent/min under the reaction conditions described above. The amount of glucose was determined using the F-kit D-glucose (JK-International, Tokyo).

2.7. The Analysis of Laminari-Oligosaccharides by Thin-Layer Chromatography (TLC)

Laminari-oligosaccharides produced by the ULam111 were analyzed by thin-layer chromatography (TLC). The oligosaccharides were developed on a TLC-60 plate (Merck, Darmstadt, Germany) with a solvent consisting of 1-butanol/acetic acid/water or ethyl acetate/acetic acid/water (2:2:1, v:v:v) and detected by spraying 10% (v/v) sulfuric acid in ethanol, followed by heating at 110˚C - 130˚C for 5 - 10 min.

2.8. The Continuous Saccharification of Laminarin by the Recirculation Batch Method

We performed the continuous saccharification of laminarin from *E. bicyclis* by the recirculation batch method [29]. To immobilize laminarinase, we mixed 2.0 mL of ULam111 (36 unit) with 500 mg of the beads in 10 mM sodium phosphate buffer (pH 6.0) and incubated the mixture at 4˚C overnight. After the incubation, the supernatant was removed and unbound protein was washed away with 50 mL of the same buffer. These experimental procedures were performed using an empty column (Econo-Column®; 185 × 18 mm; Bio-Rad). The column containing ULam111-immobilized beads was equipped with a bottle that contained 100 mL of 30 mg/mL laminarin solution added to 50 μg/mL ampicillin. The laminarin digestion reaction was performed at 30˚C for 20 hr while the laminarin solution was circulated by a peristaltic pump at 3.3 mL/min. After 20 hr, the column was washed with 50 mL of 10 mM sodium phosphate buffer (pH 6.0), equipped with a new bottle containing 100 mL of 30 mg/mL laminarin solution, and used for a second time. The above process was repeated 10 times.

2.9. Ethanol Fermentation

The ethanol fermentation was carried out by using *Saccharomyces cerevisiae* strain C-19, which was isolated from coastal water from Tokyo Bay in Japan [30]. The C-19 was incubated in 10 mL of 30 mg/mL laminarin solution digested by the immobilization reactor under anaerobic conditions at room temperature for 1 day. After the yeasts were removed by centrifugation, the amounts of ethanol were analyzed using an LC-20 HPLC system (Shimadzu, Kyoto, Japan) equipped with a refractive index detector (RID-10A; Shimadzu) through a Shim-pack SPR-Pb column (250 × 7.8 mm; Shimadzu) at 80˚C in distilled water at 0.6 mL/min.

2.10. Technical Terms and the Data Processing Methods

Efficient immobilization of ULam111 for carrier materials was evaluated according to following the equations and terminology in reference [14]: "Immobilization yield" (%) = 100 × ("immobilized activity"/"starting activity"), "Immobilization efficiency" (%) = 100 × ("observed activity"/"immobilized" activity), "Activity recovery" (%) = 100 × ("observed activity"/"starting activity"). The "immobilized activity" was determined by measuring the total residual enzyme activity that remains in the enzyme solution after immobilization and by subtracting this activity from the total "starting activity" (enzyme activity before immobilization). The "observed activity" was determined by measuring the activity of immobilized beads. Ethanol productivity (E.P.) was defined as follows: E.P. = (real ethanol production/ideal ethanol production) × 100 (%) [31].

3. Results and Discussion

3.1. The Enzymatic Properties of ULam111

A gene (*ulam*111) encoding β-1,3-glucanase-like protein, which consists of 753 bp, was found on the *Flavobacterium* sp. strain UMI-01 draft genome sequence. According to our BLAST search [32], the deduced amino acid sequence of ULam111 showed significant identity with other β-1,3-glucanases belonging to the GH16 family. As shown in **Figure 1**, the amino acid sequence of ULam111 had the highest identity (70%) with that of *Flavobacterium* sp. ACAM 123 laminarinase and relative high identity with a *Flavobacterium* sp. 4221 GH16 family precursor (68%) [33], *Cellulophaga algicola* DSM 14237 glucan endo-1,3-beta-D-glucosidase (57%) [34], a *Pedobacter* sp. 4236 GH16 family precursor (37%) [33], and *Lysobacter enzymogenes* strain N4-7 beta-1,3-glucanase A (33%) [35].

The proposed catalytic residues [37] Glu134 and Glu139 were conserved in ULam111 and the proteins listed in **Figure 1**. Since N-terminal 17 residues were predicted as a secretion signal peptide by the SignalP program [38], residues 18 - 251 of ULam111 were expressed as a recombinant protein (ULam111) with octahistidine-tag at C-terminus. ULam111 was successfully expressed and purified with the yield of 0.6 mg from 1 L of culture medium (**Figure 2**).

We examined the enzymatic properties of ULam111 using laminarin from *L. digitata*. Purified ULam111 showed degradation activity against laminarin with the specific activity of 224 ± 18 U/mg at 30˚C, pH 6.0. Its optimum temperature

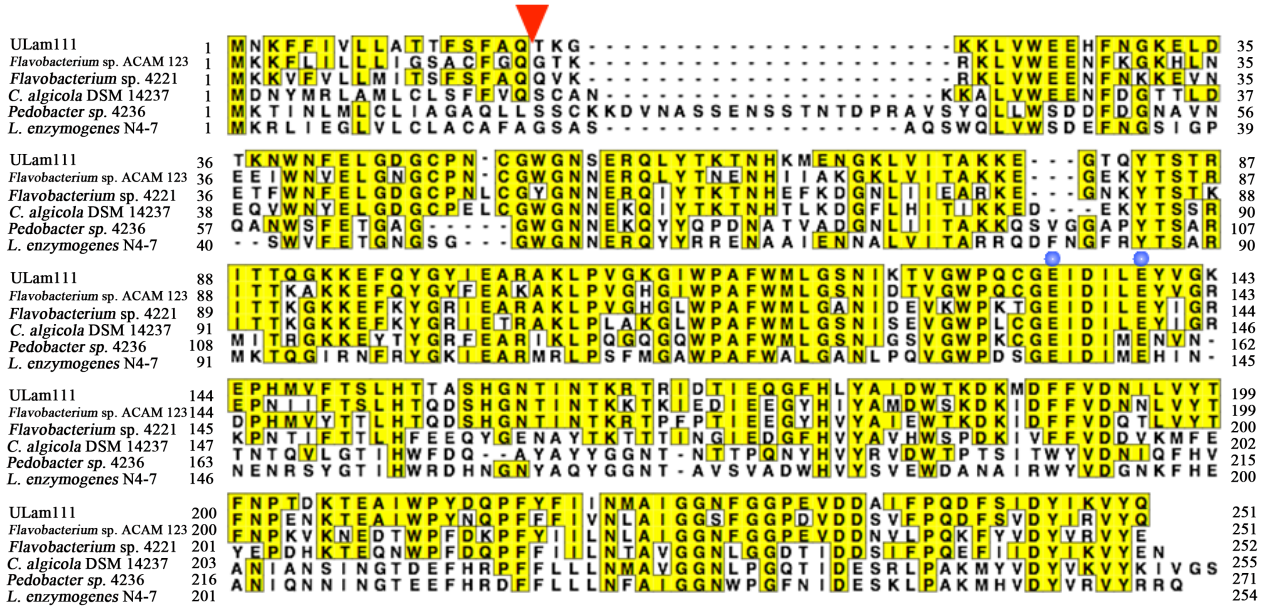

Figure 1. Multiple alignment between ULam111 and the homologous proteins. ULam111, *Flavobacterium* sp. UMI-01 candidate *β*-1,3-glucanase (in this study); *Flavobacterium* sp. ACAM 123, *Flavobacterium* sp. ACAM 123 laminarinase (GenBankacc.no. WP_ 016988671); *Flavobacterium* sp. 4221, *Flavobacterium* sp. 4221 GH16 precursor (GenBankacc.no. ABW02990, [33]); *C. algicola* DSM 14237, *Cellulophaga algicola* DSM 14237 glucan endo-1,3-beta-D-glucosidase (GenBankacc.no.YP_004163306, [34]); *Pedobacter* sp. 4236, *Pedobacter* sp. 4236 GH16 precursor (GenBankacc.no. ABW02990, [33]); *L. enzymogenes* strain N4-7, *Lysobacter enzymogenes* strain N4-7 beta-1,3-glucanase A (GenBankacc.no. AY157838, [35]). The amino acid sequences were aligned using the Clustal W program [36]. The red inverted triangle indicates a putative signal peptide cleavage site. The blue circles show putative catalytic residues.

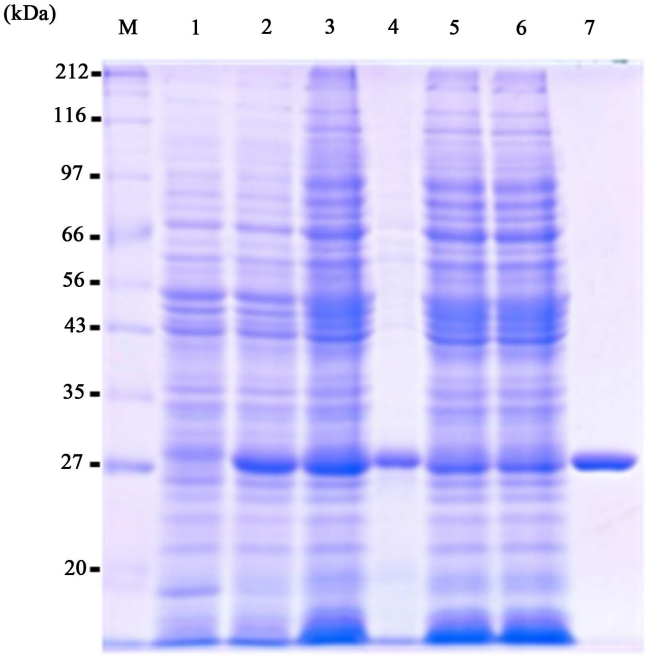

Figure 2. Bacterial expression and purification of recombinant ULam111. M; protein molecular marker, lane 1 and lane 2; cell extracts before and after induction by IPTG, respectively, lane 3; total cell extracts homogenized with a buffer containing lysozyme, lane 4 and lane 5; precipitates and supernatant after centrifugation of total fraction, respectively, lane 6; unbound fraction with resin, lane 7; purified ULam111.

was 50˚C, and degradation activities were observed at 4˚C - 80˚C (Figure 3(a)). Heat stability was investigated by incubation for 30 min at various temperatures. The remaining activity was significantly decreased by the incubation above 50˚C, and a 50% loss of activity was observed at 46˚C (Figure 3(b)). The NaCl concentration did not have a detectable effect on the activity. The highest activity was observed at 125 mMNaCl, but 72% - 78% maximal activities were measured at 500 - 1000 mMNaCl (Figure 3(c)). As shown in Figure 3(d), ULam111 preferred acidic conditions. Although its optimum pH was around 6.0, there was no activity under pH 4.0 (Figure 3(d)).

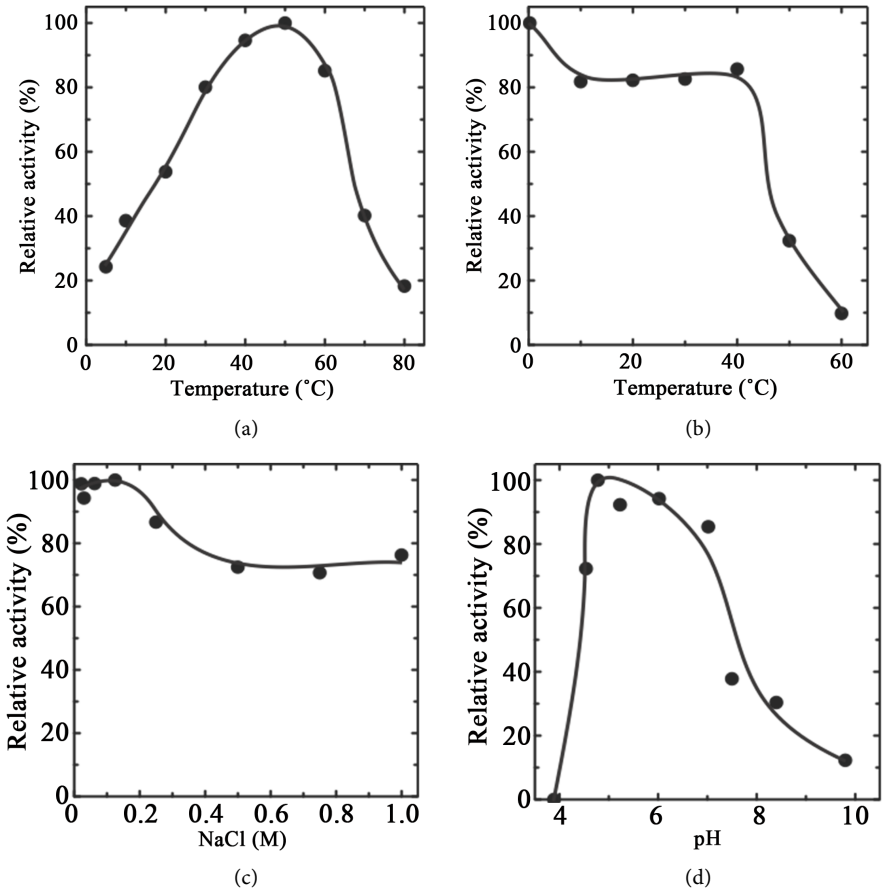

Figure 3. Effects of temperature, pH and NaCl concentration. (a) The enzyme reaction was carried out in a solution containing 10 mM sodium phosphate buffer (pH 6.0), 0.1 M NaCl, 0.1 mg/mL BSA, 2.0 mg/mL laminarin, and 0.001 mg/mL ULam111 at the indicated temperature for 15 min; (b) enzyme solution was pre-incubated at the indicated temperature for 30 min. The reaction was then carried out in a solution containing 10 mM sodium phosphate buffer (pH 6.0), 0.1 M NaCl, 0.1 mg/mL BSA, 2 mg/mL laminarin, and 0.001 mg/mL ULam111 at 30˚C for 15 min; (c) the enzyme reaction was carried out in a solution containing 10 mM sodium phosphate buffer (pH 6.0), 0.02 - 1.0 M NaCl, 0.1mg/mL BSA, 2.0 mg/mL laminarin, and 0.001 mg/mL ULam111 at the indicated temperature for 15 min; (d) the enzyme reaction was carried out in a solution containing 0.1 M NaCl, 0.1 mg/mL BSA, 2.0 mg/mL laminarin, and 0.001 mg/mL ULam111 at the indicated pH values (10 mM CH3COONa for pH 3.8 - 5.2, 10 mM sodium phosphate buffer for pH 6.0 - 7.4, 10 mM Tris-HCl for pH 8.3, 10 mM glycine-NaOH for pH 9.8) at 30˚C for 15 min.

The degradation products of laminarin by ULam111 were sequentially analyzed by TLC (Figure 4(a)). After 2 min of reaction, glucose and oligosaccharides were detected. The levels of glucose and laminaribiose increased and were identified as major products after 48 hr. Interestingly, a spot showing abnormal mobility between laminaritriose and laminaritetraose appeared after 1 hr. This product may contain not only β-1,3-bonds but β-1,6-bonds because laminarin from *L. digitata* contains β-1,3 and β-1,6 glycosidic bonds at the ratio of 7:1 [5].

We also investigated the degradation of various laminarioligosaccharides by ULam111. Among the tested oligosaccharides, only laminaribiose was undegradable (Figure 4(b)). Laminaritriose, laminaritetraose, and laminaripentaose were each degraded, and the main products were glucose and laminaribiose in each case (Figure 4(b)). Thus, trisaccharide was the minimum length for the hydrolysis of ULam111.

Next, the yield of glucose from laminarin by ULam111 was measured (Figure 5). The glucose concentration in a mixture containing 5.0 mg/mL laminarin at the start of the reaction reached a maximum at 2.5 mg/mL after 10 hr. Thus, laminarin was converted to glucose by ULam111 with 50% efficiency.

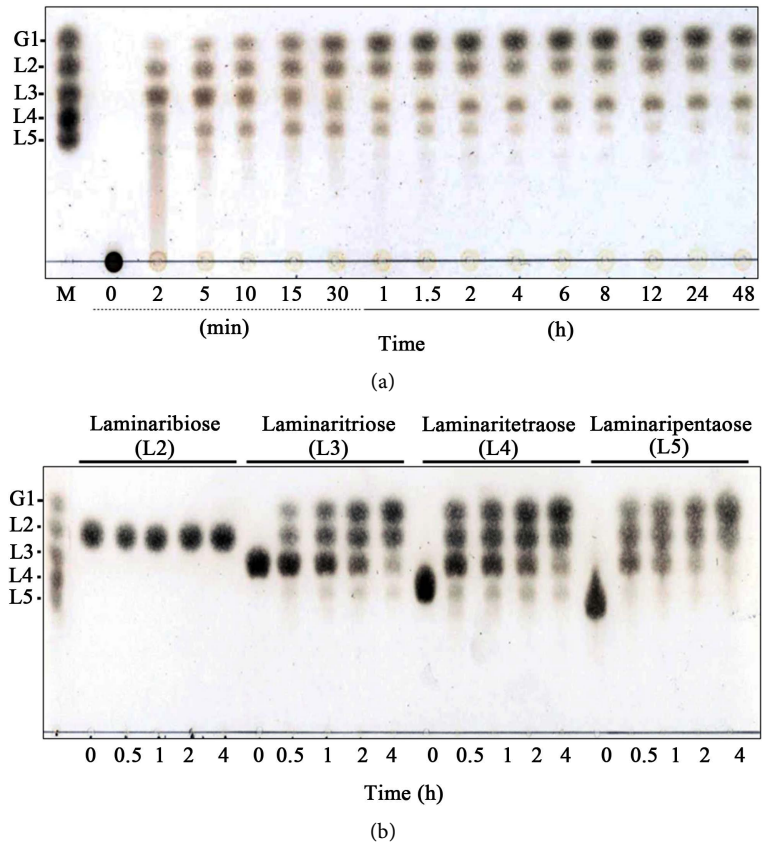

Figure 4. TLC analysis. (a) The mode of action for laminarin degradation by ULam111. The enzyme reaction was carried out in a solution containing 10 mM sodium phosphate buffer (pH 6.0), 0.1 M NaCl, 0.1 mg/mL BSA, 2.0 mg/mL laminarin, and 0.02 mg/mL ULam111-full (residues 1 - 251) or 0.001 mg/mL ULam111 at 30˚C, and then stopped at each indicated time by heating in boiling water for 10 min; (b) Degradation of laminarioligosaccharides by ULam111.

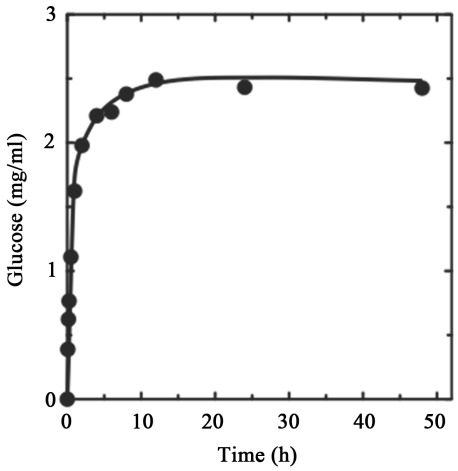

Figure 5. The amount of glucose produced by ULam111 from laminarin. The reaction was carried out in a solution containing 10 mM sodium phosphate buffer (pH 6.0), 0.1 M NaCl, 0.1 mg/mL BSA, 0.01 mg/mL ULam111, and 5.0 mg/mL laminarin at 30˚C. The amount of glucose was determined using the F-kit D-glucose (JK-International, Tokyo).

3.2. The Selection of Immobilization Beads for ULam111

To select immobilization beads suitable for ULam111 by a first screening, we investigated the "immobilization yield", "immobilization efficiency" and "activity recovery" when the 1.12 unit of laminarinase was immobilized with the use of the 11 types of commercially available immobilization beads (Table 1). First, to determine the percentage of protein bound to the immobilization beads (immobilization yield), we measured the enzyme activities in the supernatant after immobilization. Among the 11 types of immobilization beads, the seven types, *i.e.*, IB-150P (the covalent type), IB-C435 (the cationic type), IB-A369 (the anionic type), IB-EC1, IB-S861, IB-S500, IB-S60P, and IB-S60S (the non-ionic type) showed ≥ 72.9% immobilization yield. Next, to identify the percentage of remaining enzyme activities of bound protein to immobilization beads (*i.e.*, the immobilization efficiency), we measured the enzyme activities of the immobilized beads. The highest values of immobilization efficiency, were obtained with IB-S60P (the non-ionic type) at 26.6%, along with 15.6% for IB-S60S (the non-ionic type), 13.6% for IB-150P (the covalent type) and 7.71% for IB-C435 (the cationic type). Finally, we investigated the percentage of remaining enzyme activities in the immobilization beads of total protein used for the immobilization (*i.e.*, the activity recovery). The values were as follows: the highest values were 19.4% for IB-S60P (the non-ionic type), 15.6% for IB-S60S (the non-ionic type), 11.9% for IB-150P (the covalent type), and 7.1% for IB-C435 (the cationic type). We thus selected these four types of beads in the first screening.

To assess the operational stability of immobilized ULam111 on these types of four beads, we performed a sixth repeat of the batch experiments with 50 mg beads immobilizing 1.12 units of laminarinase at 30˚C for 30 min. As shown in Figure 6, for the non-ionic types IB-S60S and IB-S60P, the "observed activities" of the beads remained at 43.4% and 27.8%, respectively of the first time during the sixth cycle. In contrast, for the covalent-type IB-150P beads and the cationic-

Table 1. Immobilization evaluation of ULam111 for different carrier materials.

Product Name	Type	Matrix	Functional group	Observed activity[a] (U)	Activity in supernatant[a] (U)	Immobilization Yield (%)	Immobilization Efficiency (%)	Activity recovery (%)
IB-150P	covalent	polyacrylic	epoxide, polar	0.134 ± 0.025	0.137 ± 0.003	87.7	13.6	11.9
IB-D152	cationic	polyacrylic	carboxylic acid	0.001 ± 0.001	0.539 ± 0.069	51.8	0.2	0.1
IB-C435	cationic	polyacryic	carboxylic acid	0.079 ± 0.005	0.091 ± 0.006	91.8	7.7	7.1
IB-A161	anionic, strong	polystyrene	quat. Ammon	0	0.738	34.1	0	0
IB-A171	anionic, strong	polystyrene	quat. Ammon	0	0.704	37.1	0	0
IB-A369	anionic, weak	polystyren	quat. Ammon	0	0.082	92.9	0	0
IB-EC1	non-ionic	polyacryic	carboxylic ester	0	0.286	74.5	0	0
IB-S861	non-ionic	polystyrene	aromatic	0	0.118	89.1	0	0
IB-S500	non-ionic	polypropylene	alkyl	0	0.146	86.7	0	0
IB-S60P	non-ionic	silica, porous	hydroyl	0.217 ± 0.007	0.304 ± 0.011	72.9	26.6	19.4
IB-S60S	non-ionic	silica, super porous	hydroyl	0.174 ± 0.029	0	100	15.6	15.6

[a]The reactions were carried out in triplicate except the enzymatic activity in supernatant of carrier beads showed no "observedactivity", and the data are mean ± SD.

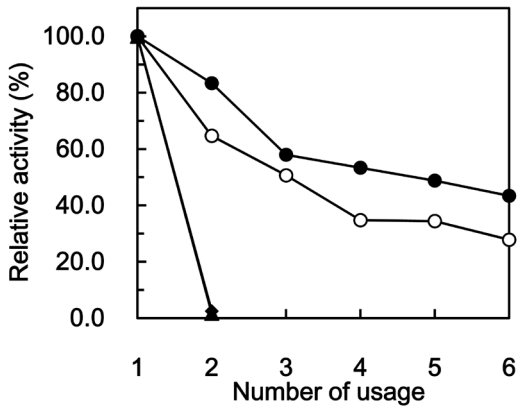

Figure 6. The relative activities of immobilized laminarinase. Each number of repetitions is shown by ○: IB-S60P (the non-ionic type), ●: IB-S60S (the non-ionic type), ♦: IB-150P (the covalent type), ▲: IB-C435 (the cationic type). One hundred percent of activity corresponds to the "observed activity" of the first time for each carrier beads.

type IB-C435 beads, during the second repetition, no "observed activity" was detected.

We therefore selected IB-S60S beads for laminarinase from among the 11 types of commercially available immobilization beads as suitable beads. IB-S60S and IB-S60P beads are solid support of silica. The silica-binding peptide mediates the efficient immobilization of each enzyme onto mesoporous silica-based materials such as zeolite for thermostable hemicellulases: β-glucosidase, β-xylanase and β-mannanase [39]. Glycoside hydrolases including laminarinase often have non-catalytic carbohydrate-binding modules (CBMs) appended to the catalytic domain [40]. Several studies have used a CBM as an affinity tag for en-

zyme immobilization to solid support made from carbohydrate, and in some instances, the enzymatic activity was increased [41]. If we could identify a linker adapted to ULam111 such as a silica-binding peptide, we may be able to increase the enzymatic activity of immobilized ULam111.

3.3. The Continuous Saccharification of Laminarin by Immobilized ULam111 Followed by Ethanol Fermentation

For the saccharification of a large volume of laminarin solution, we constructed an experimental reactor system with immobilized ULam111 using the non-ionic type IB-S60S beads, as shown in **Figure 7**. In this reactor system, approx. 10 - 18 g/L glucose was produced from 30 mg/mL laminarin solutions after 20 hr, when the reactor operation was repeated 10 times (**Figure 8(a)**). When free laminarinase with an equal or one-tenth amount of immobilized enzyme was used in one trial, 23.2 g/L or 13.0 g/L of glucose was produced, respectively. Therefore, the immobilized laminarinase was found to retain sufficient activity during repeated trials in the reactor system.

To analyze the digestion product, we performed a TLC analysis of the saccharification product. As shown in **Figure 8(b)**, the production of glucose was confirmed in 10 repetitions, but the amount of glucose gradually decreased. Spots of triose most strongly appeared. These spots also had abnormal mobility between laminaritriose and laminaritetraose. More interestingly, although ULam111 did not have the ability to digest laminaribiose (**Figure 4(b)**), laminaribiose (L2) was not detected. These products might have contained many trioses and may not have contained laminaribiose because laminarin from *E. bicyclis* has β-1,6 glycosidic bonds at very high rates (β-1,3: β-1,6 = 3:2) [6]. Therefore, providing hydrolysis enzymes that are capable of the digestion of β-1,6-linked glucose branches and laminaribiose may significantly improve the glucose yield.

We also measured the production of reducing sugars (**Figure 8(c)**) and glucose (**Figure 8(d)**) over time. The glucose 1.2 - 1.9 g/L was repeatedly produced from 30 mg/mL laminarin solutions after 20 hr when the reactor was operated 10

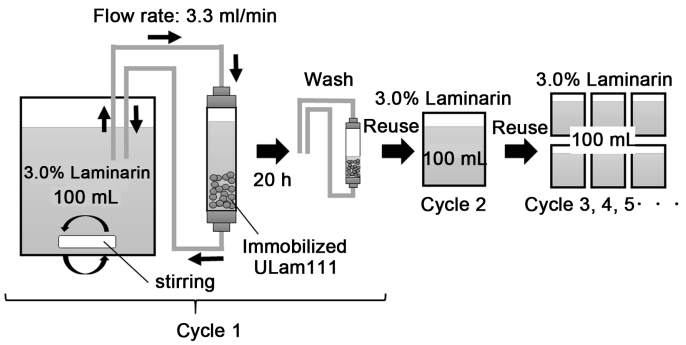

Figure 7. The schema of the experimental reactor system using immobilized laminarinase. The laminarin digestion reaction was performed at 30˚C for 20 hr while a laminarin solution was circulated by a peristaltic pump at 3.3 mL/min. After 20 hr, this column was washed with 50 mL of 10 mM sodium phosphate buffer (pH 6.0), equipped with a new bottle containing 100 mL of 30 mg/mL laminarin solution, and used for the second time. The above process was repeated 10 times.

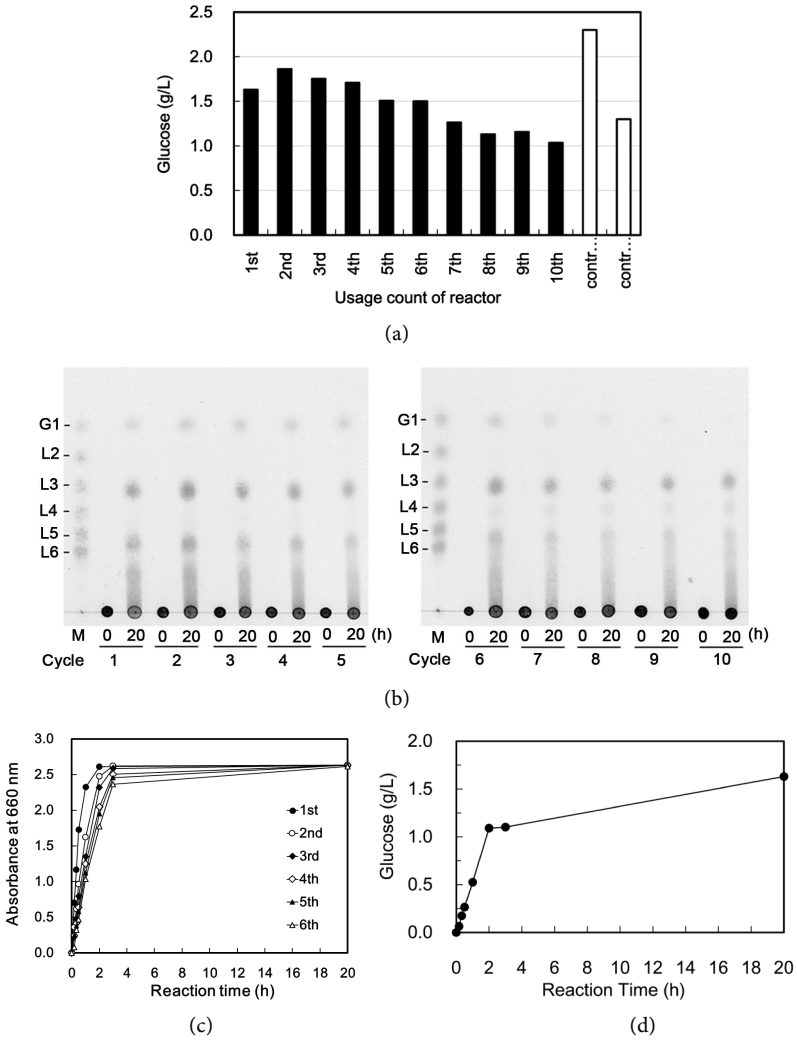

Figure 8. Continuous saccharification of laminarin by ULam111-immobilized IB-S60S beads. (a) The amount of glucose production by the experimental reactor system at each number of repetitions; (b) the TLC analysis of laminari-oligosaccharides by the experimental reactor system at each number of repetitions; (c) the production of reducing sugar over time by the experimental reactor system at each number of repetitions; (d) production of glucose overtime.

times. The production of reducing sugar was terminated for approx. 3 hr and the amounts of reducing sugar were almost equal in the 10-times trials of the reactor. As shown in **Figure 8(d)**, nearly 70% of the glucose was produced within 2 hr reaction time. Our results thus demonstrated that most of the glucose and oligosaccharide was produced within 2 - 3 hr using this reactor, but the glucose yield increased by using this reactor for a longer time.

Following the saccharification of laminarin by immobilized ULam111, we examined the ethanol fermentation using the second and fourth saccharified solutions of 10-times saccharification. The results showed that 0.58 g/L (60.1% of the theoretical value) and 0.51 g/L (58.3% of the theoretical value) were produced from laminarin saccharified solutions that contained 1.86 g/L and 1.71 g/L of glucose for 20 hr, as shown in **Table 2**.

Table 2. Ethanol production from 30 mg/mL laminarin saccharified solutions by a marine-derived yeast *S. cerevisiae* C-19.

No. of saccharified solutions[a]	Glucose[b] (g/L)	Ethanol (g/L)	Ethanol productivity (%)
2nd	1.86	0.58	61.0
4th	1.71	0.51	58.3

[a]Saccharified solutions were produced during this number of repetitive cycles of the reactor system with ULam111 immobilized on IB-S60S. [b]The concentration of glucose inlaminarin saccharified solutions produced using the reactor system.

4. Conclusion

We investigated the continuous saccharification of laminarin by immobilized laminarinase ULam111 followed by ethanol fermentation with a marine-derived yeast. The novel laminarinase ULam111 was isolated from *Flavobacterium* sp. UMI-01, and purified ULam111 showed degradation activity against laminarin with the specific activity of 224 ± 18 U/mg at 30˚C and pH 6.0. With the use of our reactor system with ULam111 immobilized on IB-S60S carrier beads, 1.2 - 1.9 g/L glucose was repeatedly produced from 30 mg/mL laminarin solutions after 20 hr when the reactor was operated 10 times. Ethanol fermentation from the saccharified solutions with the marine-derived yeast *S. cerevisiae* C-19 was carried out, and 0.51 - 0.58 g/L bioethanol was produced from the saccharified solution containing 1.71 - 1.86 g/L of glucose.

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