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A MALDI-MS-based Glucan Hydrolase Assay Method for Whole-cell Biocatalysis

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Screening microorganisms that can produce glucan hydrolases for industrial, environmental, and biomedical applications is important. Herein, we describe a novel approach to perform glucan hydrolase screening—based on analysis of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) spectra—which involves degradation of the oligo- and polysaccharides. As a proof-of-concept study, glucan hydrolases that could break down glucans made of several glucose units were used to demonstrate the MALDI-MS-based enzyme assay. First, the enzyme activities of α-amylase and cellulase on a mixture of glucan oligosaccharides were successfully discriminated, where changes of the MALDI-MS profiles directly reflected the glucan hydrolase activities. Next, we validated that this MALDI-MS-based enzyme assay could be applied to glucan polysaccharides (*i.e.*, pullulan, lichenan, and schizophyllan). Finally, the bacterial glucan hydrolase activities were screened on 96-well plate-based platforms, using cell lysates or samples of secreted enzyme. Our results demonstrated that the MALDI-MS-based enzyme assay system would be useful for investigating bacterial glucoside hydrolases in a high-throughput manner.

Keywords: **Glucan hydrolase, glucan, oligosaccharides, polysaccharides, MALDI-MS, whole-cell biocatalysis**

Introduction

Glucan hydrolases (*i.e.*, glucanase) hydrolyze glucosidic linkages in the oligo- or poly-saccharides according to their substrate specificity [1]. Due to their various biological activities, glucan hydrolases have biomedical, industrial and environmental applications, including pathogen defense [2], biomass degradation [3, 4] and organic waste recycling process [5–7]. Microorganisms are tremendous sources of glucan hydrolases. In addition, target enzymes can be produced with inexpensive methods such as large-scale fermentation processes using genetically engineered microbes [8]. Therefore, screening microorganisms to identify enzymes with novel activities is very important to develop commercial biological processes for the production of valuable chemicals [9], pharmaceuticals [10] and biofuels [11].

General bacterial enzyme screening methods have widely used chromogenic and fluorogenic substrates. In such screening methods, bacterial enzyme activities are directly characterized by changing the color or fluorescence of a particular substrate compound [12–14]. These substrates have been applied to screen enzymes such as protease [15], lipase [16] and glucosidase [17, 18]. However, such methods ineluctably require expensive reagents and complex synthetic processes for chromophore or fluorescence-labeled substrates. In addition, they are barely applicable to high-throughput platforms due to high cost of synthetic substrates. Alternatively, mass spectrometry can be used as a label free tool to identify bacterial enzyme activity [19]. Gas chromatography-mass spectrometry (GC-MS) and liquid chroma-

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tography-mass spectrometry (LC-MS) could be applied to screen bacterial enzyme activities in a quantitative manner. For example, the activity of bacterial carbohydrate sulfotransferase has been analyzed by LC-MS using label-free method [20]. Glucan hydrolase activity from bacteria (e.g., *Escherichia coli* K12, *Bacillus cereus* and *Pseudomonas aeruginosa*) has also been analyzed by LC-MS using a mixture of substrates [21]. However, GC-MS or LC-MS based bacterial enzyme screening methods involve many complex manual steps with long turnaround time [22]. To overcome such problems, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-MS) could be a good candidate to detect enzyme activities (e.g., lipase [23], β-lactamase [24] in bacterial lysates). MALDI-MS inherently has the advantages of rapid turnaround time and low-cost consumables which are beneficial characteristics for high-throughput assay platform.

Here, we describe a MALDI-MS based bacterial enzyme screening method to detect glucan hydrolase activities and specificities using unlabeled glucan substrates, including glucan oligosaccharides and polysaccharides (Table 1). To validate such MALDI-MS-based enzyme assay, each α-amylase and cellulase was examined with glucan oligosaccharides (*i.e.*, maltoheptaose, cellohexaose). We demonstrated that this assay could enable us to identify glucan hydrolase activities even when a mixture of oligomer substrates was used. Next, we showed that this MALDI-MS-based enzyme assay could be applied to glucan polysaccharides (*i.e.*, pullulan, lichenan, schizophyllan). Finally, it could be employed for screening a glucan hydrolase against the bacterial lysates and the samples of secreted enzyme. Such MALDI-MS-based enzyme assay system could be applied to biomass degradation and high throughput screening of bacterial glucan hydrolase to produce industrially and environmentally useful carbohydrate monomers.

Table 1. List of glucan substrates and enzyme used in study.

Substrate	Enzyme	Linkages
Maltoheptaose	α-Amylase	α-1,4-glucosidic linkages
Cellohexaose	Cellulase	β-1,4-glucosidic linkages
Pullulan	α-Amylase	α-1,4-glucosidic linkages
Schizophyllan	endo-1,3-β-D-glucanase	β-1,3-glucosidic linkages branched-β-1,6-linkages
Lichenan	Lichenase	β-1,3 and -1,4-glucosidic linkages

Materials and Methods

Materials

Super 2,5-Dihydroxybenzoic acid (Super-DHB), cesium iodide (CSI), trans-2-(3-(4-tert-Butylphenyl)-2-methyl-2-propenylidene] malononitrile (DCTB), acetonitrile, and distilled water were purchased from Sigma-Aldrich (USA). Methanol was obtained from Junsei Chemical (Japan). α-Amylase, lichenase, endo-1,3-β-D-glucanase, cellulase, lichenan, and pullulan were purchased from Megazyme (USA). Maltoheptaose and cellohexaose were purchased from Toronto Research Chemicals (Canada). Schizophyllan was obtained from Quegen (Korea).

Enzyme assays

Maltoheptaose was dissolved in 0.02 M sodium phosphate buffer (pH 6.5) to obtain concentration of 0.2 wt%. The solution was mixed with α-amylase (8 U/mg). The mixture was then incubated at 40℃ for 16 h. Likewise, cellohexaose was dissolved in 0.02 M sodium citrate buffer (pH 4.5) to obtain a concentration of 0.2 wt%. Cellulase (8 U/mg) were added to the solution and then the mixture was incubated at 40℃ for 16 h. Pullulan was dissolved in 0.02 M sodium phosphate buffer (pH 6.5) to reach a concentration of 2 wt%. Lichenan was dissolved in 0.02 M sodium citrate buffer (pH 4.5) at a concentration of 2 wt%. Schizophyllan was dissolved in 0.02 M sodium citrate buffer (pH 4.5) at a concentration of 0.4 wt%. Enzyme reaction was stopped by incubating at 90℃ for 10 min. The sample was then dried. Dried sample was dissolved in 10 μl of 50% methanol/water (v/v).

MALDI-MS analysis

Super-DHB matrix was dissolved in 70% acetonitrile/water (v/v) to obtain concentration of 50 mg/ml. After 1 μl of the sample was mixed with 1 μl of Super-DHB matrix solution, the mixture was spotted onto a stainless steel MALDI plate and dried at room temperature. Analysis of enzyme activity was performed using Microflex LRF MALDI and Bruker UltrafleXtreme in reflectron mode (Bruker, Germany). MALDI mass spectra were acquired with a total of 1000 shots from five different spots in positive reflectron mode. The following instru-

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ment settings were used: accelerating voltage = 20 kV, laser frequency = 60 Hz, ion source 1 voltage = 19 kV, ion source 2 voltage = 16 kV, lens voltage = 9.8 kV, detector gain = 5.8, and laser power = 85–90%. The UltrafleXtreme was operated with the following settings: NDL YAG (solid state) laser at 355 nm utilizing SmartBeam modulation; extraction voltage, 10 kV; delay time, 70 ns. Spectral acquisition and processing were performed with Flex Analysis software (ver. 3.3, Bruker).

96-Well plate-based glucan hydrolase activity screening

Escherichia coli O157 strains ATCC 700927 and *Escherichia coli* K12 strain w3110 were kindly provided from Prof. Byung-Gee Kim (Seoul National University, Korea). *Pseudomonas aeruginosa* strain KCTC 1637, *P. syringae* strain KCTC 1832, *Bacillus substilis* strain KCTC 1021, *Bacillus cereus* strain KCTC 1012 were obtained from the Korean Collection for Type Cultures (KCTC) Biological Resource Center (Korea). *Salmonella enterica subsp.* strains Minnesota CCARM 0044, *Enteritidis* strain CCARM 0134, *Typhimurium* strain CCARM 0125 were obtained from the Culture Collection of Antibiotic-Resistant Microbes (CCARM) at Seoul Women's University (Korea). The cells were cultured in 50 ml nutrient medium at 37℃ for 24 h with shaking at 200 rpm. *Escherichia coli* O157 and *Escherichia coli* K12 were cultured in 50 ml LB medium at 37℃ for 24 h with shaking at 200 rpm. These cells were harvested by centrifugation at 4,000 rpm for 15 min at 4℃. After transferring the supernatant into new tubes, cell pellet was resuspended in 20 ml of distilled water and then centrifuged at 4,000 rpm for 15 min at 4℃ as a washing step. This step was repeated again. The cell pellet was resuspended in 5 ml of 20% (v/v) glycerol. The cells were lysed by cell sonication using an Ultrasonicator (Sonics & Materials, USA) for 4 cycles (5 s pulse, 30 s pause) at an amplitude of 25%. The equivalent volume of maltoheptaose and cellohexaose were mixed with the identical concentration (0.2 wt%). Each 10 μl of cell lysate or supernatant medium was mixed with 20 μl of the glucan oligosaccharide substrates, 150 μl of distilled water,

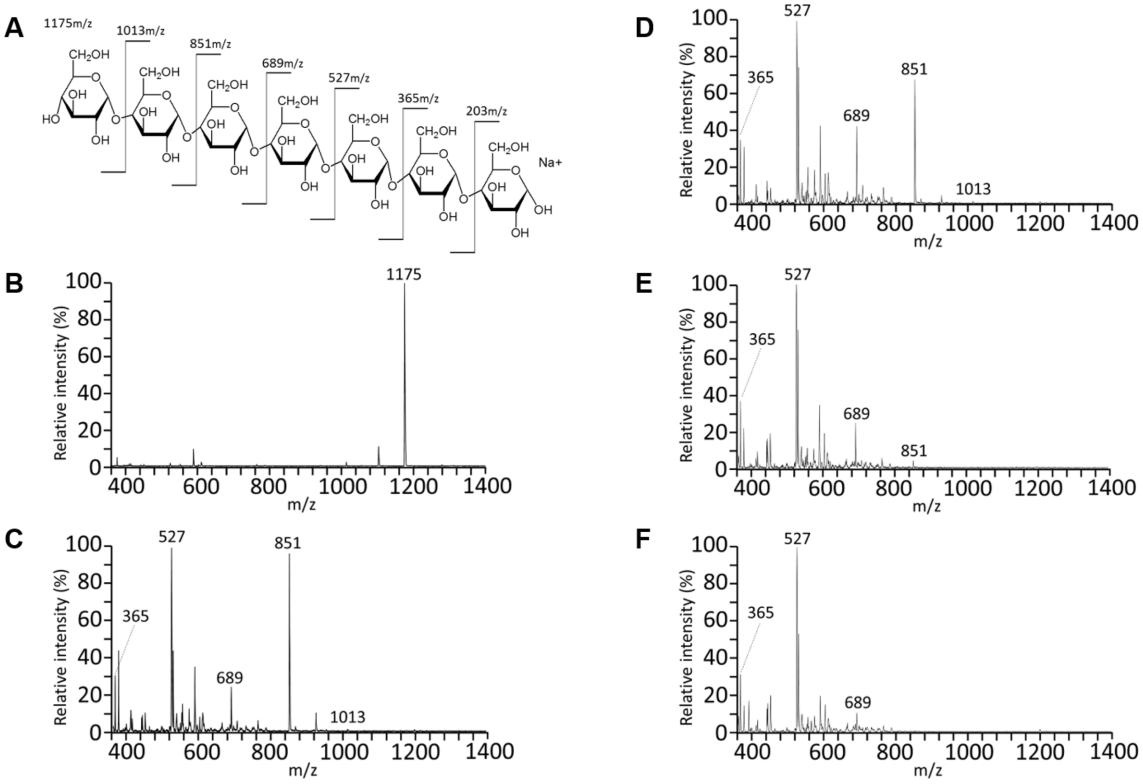

Fig. 1. (A) Structure of maltoheptaose. A MALDI spectra of the maltoheptaose hydrolysis assay with α-amylase in time course (B) maltoheptaose (1175 *m/z*) at 0 h, (C) reaction after 1 h, (D) reaction after 2 h, (E) reaction after 6 h and (F) reaction after 16 h.

10 μl of 0.02 M sodium phosphate buffer (pH 6.5) and 10 μl of 0.02 M sodium citrate buffer (pH 4.5) in 96-well plates. The enzyme reaction was carried out at 37℃ for 6 h and then terminated at 90℃ for 10 min. The enzyme reaction products were dried for the further MALDI-MS analysis.

Results and Discussion

To verify the MALDI-MS-based enzyme assay, α- and β-glucan hydrolases were first investigated with glucan oligosaccharides. Each maltoheptaose (*i.e.*, linear α-1,4-linked glucan oligomer, Fig. 1A) and cellohexaose (*i.e.*, linear β-1,4-linked glucan oligomer, Fig. 1B) was hydrolyzed with the enzyme and the enzyme activity was then analyzed using MALDI-MS without any further purification steps (Figs. 1 and 2). Precursor ion of maltoheptaose was identified at m/z 1175 corresponding to maltoheptaose-sodium adduct form ($[M+Na]^+$) (Fig. 1B) [25]. After enzyme treatment with α-amylase, fragment ions m/z 1013, 851, 689, 527, and 365 generated from loss of hexose residues (162 mass units) confirmed the hydrolysis reaction of maltohexaose (Fig. 1). When reaction time was increased, the intensity of ion (m/z 851) was decreased. Thus, α-amylase worked. As shown in Fig. 2B, the spectrum of cellohexaose was identified as sodium adduct ion (m/z 1013) which was quite common in MALDI [26]. In addition, when the reaction time was increased, peaks of short chain fragments were dominant in MALDI profiles (Fig. 2C–F). These results demonstrate that the time course of glucan hydrolase-catalyzed reactions can be relatively monitored by MALDI-MS. Next, a mixture of maltohexaose and cellohexaose (Fig. 3A) was treated with α-amylase or cellulase. Results of hydrolysis of glucan oligosaccharides by α-amylase are shown in Fig. 3B. The intact mass of maltoheptaose consisting of α-1,4-linkage only disappeared after the enzyme treatment. However, it remained as a major sugar in the reaction of cellulase (Fig. 3C). These results showed that the enzyme activity of glucan hydrolase could be characterized by MALDI-MS-based enzyme assay.

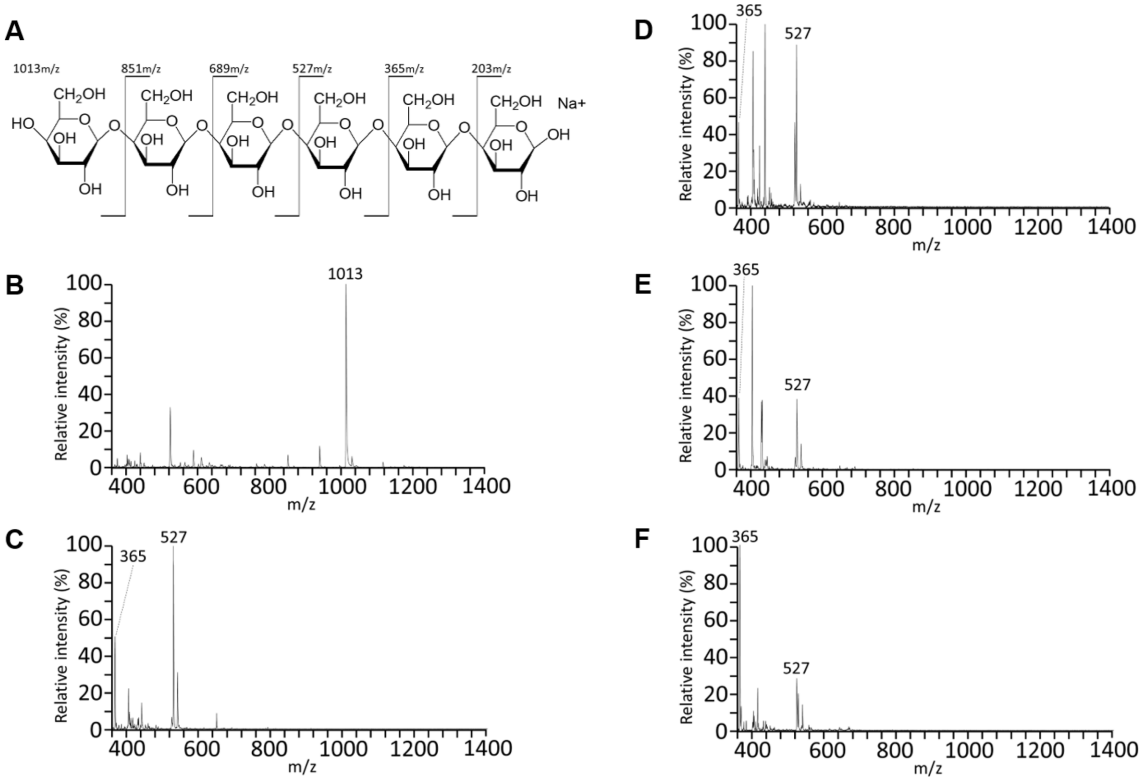

Fig. 2. (A) Structure of cellohexaose. A MALDI spectra of the cellohexaose hydrolysis assay with cellulase in time course (B) cellohexaose (1013 *m/z*) at 0 h, (C) reaction after 1 h, (D) reaction after 2 h, (E) reaction after 6 h and (F) reaction after 16 h.

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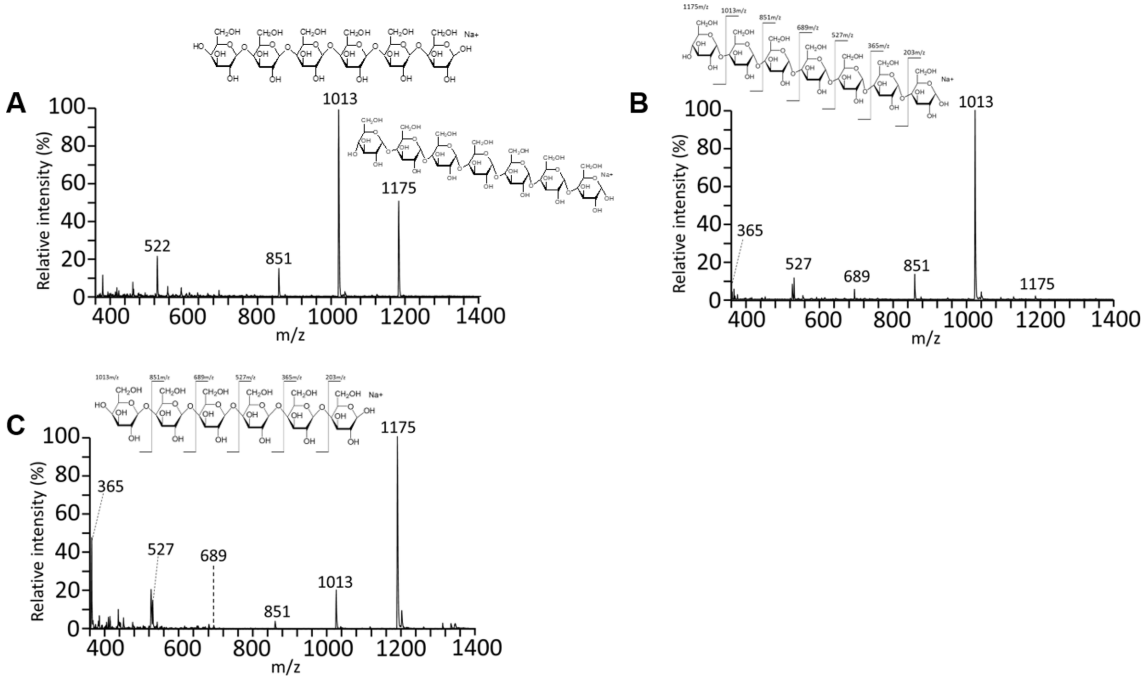

Fig. 3. MALDI spectra of (A) mixture of maltoheptaose (1175 *m/z*) and cellohexaose (1013 *m/z*), (B) the product of the mixture hydrolysis assay with α-amylase and (C) cellulase after 16 h.

We expanded the application of MALDI-MS-based assay into polysaccharides including α- and β-glucosidic linkages. Pullulan is a polysaccharide polymer which has α-1,4-linkage and α-1,6-liankge. It was used to validate changes of MALDI spectrum after treatment with α-glucan hydrolase. The MALDI spectrum of pullulan polysaccharide showed interval of *m/z* 162 units with positive ion clusters such as $[M+Na\text{-}H_2O]^+$, $[M+Na]^+$, and $[M+K]^+$ (Fig. 4A) [27–29]. After reaction with α-amylase for 16 hours, the spectrum showed that the intensity of relative low mass ion corresponding to product ion ($[M+Na]^+$) was increased as α-amylase hydrolyzed the α-1,4-glucosidic bond. Schizophyllan has β-1,3-glucan branched with β-1,6-glucosidic linkages. As shown in Fig. 5, there were differences in spectrum between before and after endo-1,3-β-D-glucanase reaction, namely hydrolysis of the β-1,3-linkage. Schizophyllan was then analyzed by MALDI-MS. Its spectrum showed peaks *m/z* 995, 1157, 1319, 1431, 1593, 1755, and 1917 due to loss of water at 18 mass unit from sodiated ions series as stated before. After the reaction, the spectrum showed more peaks of relative lower mass

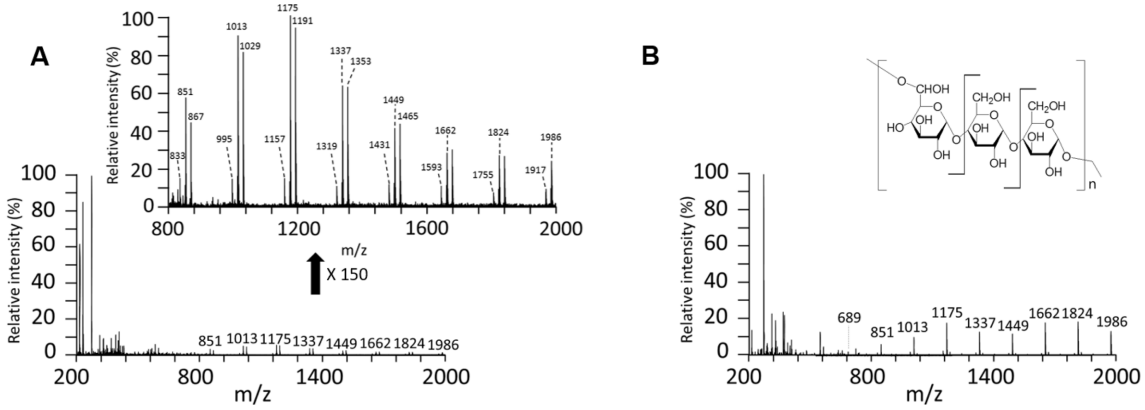

Fig. 4. MALDI spectra of (A) pullulan and (B) the products of pullulan hydrolysis with α-Amylase for 16 h.

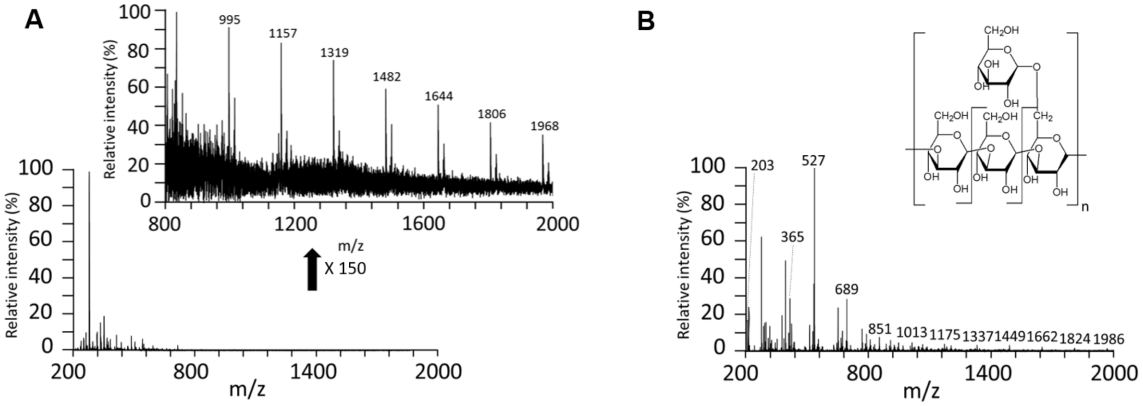

Fig. 5. MALDI spectra of (A) Schizophyllan and (B) the products of schizophyllan hydrolysis with endo-1,3-β-D-glucanase for 16 h.

([M+Na]$^+$). The most dominant peak (m/z 527) corresponded to a structure with β-1,3-linakge or branched β-1,6-linkage. Next, lichenan, a polysaccharide consisting of β-1,3 and 1,4-glucosidic linkage, was analyzed by MALDI-MS. The spectrum profile of lichenan showed difference in a glucose (162 Da) between peaks as shown in Fig. 6A. Since lichenan had two types of glucosidic linkages, lichenan reacted with endo-1,3-β-D-glucanase and lichenase. Endo-1,3-β-D-glucanase has the hydrolase activity as stated before while lichenase can hydrolyse β-1,4-glucosidic linkage containing 1,3- and 1,4-glucosidic bonds. Figs. 6B and 6C show the MALDI spectra after reacting with each enzyme. These spectra have different patterns. As shown in Fig. 6B, the dominant

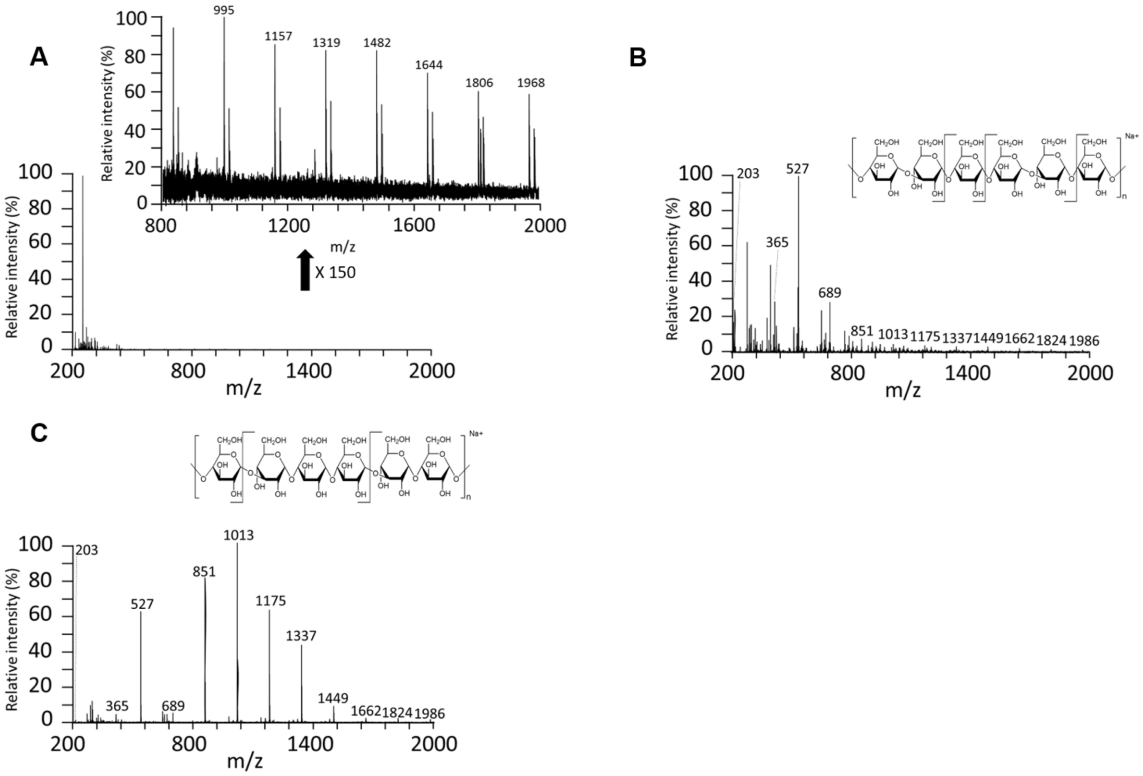

Fig. 6. MALDI spectra of (A) lichenan and products of lichenan hydrolysis with (B) endo-1,3-β-D-glucanase and (C) lichenase for 16 h.

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peak (m/z 527) is the trimer of glucose that could be obtained when all β-1,3-linkages in lichenan are hydrolyzed by endo-1,3-β-D-glucanase. On the other hand, the most abundance peak at m/z 1013 is 6 hexose residues from cleavage of lichenan while the ion at m/z 851 was caused by the loss of the terminal glucose from hexamer of glucose. These results indicate that lichenase can hydrolyze (1→4)-β-glucosidic linkages in β-glucosidic consisting of (1→3)-and (1→4)-bonds [30]. Likewise, ions (m/z 1175 and 1337) are 7 and 8 hexose residues from lichenan. They are products of lichenase with the same mechanism (Fig. 6C). These results demonstrated that polysaccharides could be used as substrates of glucosidase in MALDI-MS based screening.

With regard to industrial applications, whole-cell reaction for screening specific enzymes has a number of advantages [31–33]. Here, MALDI-MS-based enzyme assay was investigated using whole-cell biocatalysis. We used 9 bacterial strain: *E. coli* 0157, *E. coli* W3110, *P. aeruginosa, P. syringae, S. enterica* Minnesota, *S. entrica Enteritidis*, *S. enterica Typhimurium*, *B. subtilis, B. cereus, Salmonella enterica subsp. enterica serovar Enteritidis* (*S. enteritidis*), *Salmonella enterica subsp. enterica serovar Typhimurium* (*S. typhimurium*), *Bacillus substilis* (*B. subtilis*), *Bacillus cereus* (*B. cereus*). We tested whether a mixture of glucan oligosaccharides could be used to detect specific glucan hydrolase activity in the 96-well plate platform. As shown in Table 2, *B. subtilis, B. cereus, E. coli* O157 and *E. coli* K12 have both α-amylase and cellulase [34–39]. *S. entrica* strains Minesota, *Enteritidis* and *Typhimurium* have only α-amylase [40–42]. *P. aeruginosa* and *P. syringae* do not have α-amylase and cellulase. To verity the activity of α-amylase and cellulase, the mixture of glucan oligosaccharide substrates was reacted with the cell lysate or supernatant medium and their enzyme reaction products were directly analyzed by MALDI-MS. First, the cell lysates of *B. subtilis, B. cereus, E. coli* O157, *E. coli* W3110, *S. enterica minnesota*, *S. enteritidis* and *S. typhimurium* contained α-Amylase activity which remarkably decreased the maltoheptaose peak at m/z 1175 (Fig. S1 and S2). In the medium supernatants, the cellohexaose (m/z 1013) was cleaved by extracellular cellulase from *B. subtilis* and *B. cereus* (Fig. S1B and D). In addition, the maltoheptaose and the cellohexaose were not detected in the MALDI spectra of *E. coli* O157 and *E. coli* W3110 medium supernatants (Fig. S1F and H). On the other hand, there was no change in peak intensity of the both glucan oligosaccharide substrates after the enzyme reaction using cell lysates and medium supernatants from *P. aeruginosa* and *P. syringae* (Fig. S3). This suggests that MALDI-MS-based enzyme assay platform could be applied to high throughput bacterial enzyme screening using a variety of substrates or mixtures. It might be

Table 2. MALDI-MS-based bacterial glucan hydrolase activity screening in 96-well plate platform.

Microorganisms	Activity	Enzyme	Gene	Activity by MALDI-MS	Reference
Bacillus subtilis	α-1,4-glucosidic linkage	Amylase	amyE	Cell lysate	[34]
	β-1,4-glucosidic linkage	Cellulase	eglS	Medium supernatant	[35]
Bacillus cereus	α-1,4-glucosidic linkage	Amylase	BC_1157	Cell lysate	[36]
	β-1,4-glucosidic linkage	Cellulase	bcere0025_31870	Medium supernatant	
Escherichia coli O157	α-1,4-glucosidic linkage	Amylase	amyA	Cell lysate Medium supernatant	[37]
	β-1,4-glucosidic linkage	Cellulase	bcsZ	Medium supernatant	
Escherichia coli W3110	α-1,4-glucosidic linkage	Amylase	amyA	Cell lysate Medium supernatant	[38]
	β-1,4-glucosidic linkage	Cellulase	bcsZ	Medium supernatant	[39]
Salmonella enteritidis	α-1,4-glucosidic linkage	Amylase	ABA47_1293	Cell lysate	[40]
Salmonella minnesota	α-1,4-glucosidic linkage	Amylase	malS	Cell lysate	[41]
Salmonella typhimurium	α-1,4-glucosidic linkage	Amylase	malS	Cell lysate	[42]
Pseudomonas aeruginosa	N.A*	N.A		N.D*	
Pseudomonas syringae	N.A	N.A		N.D	

*N.A: not applicable, N.D: not detectable.

useful for screening other glucan hydrolases that can decompose biomass.

In conclusion, this study demonstrates a glucan hydrolase screening method based on pattern recognition of MALDI-MS spectra involving degradation of oligo- and poly-saccharides as substrates. By using α-/β-1,4-linked glucan oligosaccharides, enzyme activities of α-amylase and cellulase could be discriminated. Such MALDI-MS-based enzyme assay platform was also successfully applied when glucan polysaccharides were used as substrates. Finally, the enzyme activity in whole-cell lysate was validated by using a mixture of glucan oligosaccharides. Overall, such glucosidase screening method based on MALDI-MS is simple with high-throughput. It is valuable for characterizing linkage-/component-specific glucan hydrolase in newly discovered microorganism. Such glucosidase screening method could be applied to decompose biomass and find novel enzymes for the biochemical industry.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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