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Modification of lignin in sugarcane bagasse by a monocopper hydrogen peroxide-generating oxidase from *Thermobifida fusca*

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ABSTRACT

Agricultural lignocelluloses could represent great resources for the production of biofuels and renewable chemicals if the recalcitrant lignin polymer could be efficiently removed without negatively impacting the environment. Recently, a monocopper polyphenol oxidase (Tfu1114) from *Thermobifida fusca* was found capable of assisting the bacterium's own xylanase/cellulase to release reducing sugars from sugarcane bagasse. This study demonstrates that molecular oxygen was reduced to hydrogen peroxide by Tfu1114, coupled to the oxidation of 2,6-dimethoxyphenol, alkaline lignin, or sugarcane bagasse. Tfu1114 was able to reduce the total phenolic content of alkaline lignin and shift the molecular weight distribution to species with smaller sizes. Dilignol subunits such as syringaresinol and simulanol were generated in the hydrolysate of bagasse treated with Tfu1114. FTIR analysis suggests that Tfu1114 removed some C—C and/or C—O bonds adjacent to aromatic rings of lignin. From an applied perspective, Tfu1114 and cellulases acted in concert to release reducing sugars that were as usable as glucose for the cultivation of *Escherichia coli*, suggesting that toxins such as furfural and furan were absent, or at ignorable concentrations, in the hydrolysate. Tfu1114, therefore, represents a new type of lignin-degrading enzyme with potential to provide an eco-friendly way to modify the lignin in lignocellulose.

1. Introduction

Immense amounts of agricultural wastes such as sugarcane bagasse, corn cobs, and rice straw are disposed worldwide annually. Polysaccharides, particularly cellulose, within those lignocellulosic wastes will provide valuable resources for biofuel and chemical production if they are retrieved properly. The presence of lignin, highly recalcitrant towards chemical and biological degradation due to its molecular architecture, protects the renewable polysaccharides in lignocelluloses from being digested by cellulolytic enzymes. Many physical and chemical pretreatment processes to disrupt lignin have been published in literature [1–3]. The common methods include, but not limited to, steam and ammonia explosion, alkaline hydrolysis, and dilute acids at high temperatures. Although every method has its merits, implementation of the pretreatment may face obstacles such as formation of inhibitory byproducts (e.g. furans and furfural) for further microbial fermentation, high use of water and/or energy, and considerable production of wastes. Therefore, development of an environment-friendly process for lignin degradation is increasingly important, while the demand for fuels continues to rise in the long run.

Lignin, the third most abundant constituent of plant biomass after cellulose and hemicellulose, is a very complex and heterogeneous polymer in which different phenylpropanoid aryl-C3 units, typically including *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, form a three-dimensional network linked through diverse ether and carbon-carbon bonds [4–6]. The lignin content in lignocellulosic biomass varies from about 15–35% depending on the type of plant. White-rot basidiomycetes are the best known organisms to mineralize lignin to carbon dioxide in nature. The degradation pathways of lignin by the fungi involve both enzymatic and non-enzymatic mechanisms [7–9]. The responsible enzymes secreted by white-rot basidiomycetes include phenol oxidase (laccase), heme-containing peroxidase (lignin peroxidase, manganese peroxidase, and versatile peroxidase), and accessory

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hydrogen peroxide-generating oxidases such as aryl-alcohol oxidase and glyoxal oxidase. Concerted actions of these enzymes on lignocelluloses, in combination with a variety of redox mediators, generate small reactive oxidants, e.g. hydroxyl radicals, which attack polysaccharides and lignin in a non-specific manner. In addition, hydroxyl radicals generated from hydrogen peroxide via the Fenton reaction also contribute to the non-enzymatic lignocellulose degradation. Compared to fungi, bacteria that may break down lignin have received less attention. Sporadic reports in the literature did suggest the involvement of bacteria such as *Streptomyces* and *Pseudomonas* species in lignin degradation [10–12]. Recently, several dye de-colorizing peroxidases (Dyp-type peroxidases) from bacteria such as *Rhodococcus jostii* RHA1 and *Pseudomonas fluorescens* Pf-5 were reported to attack a β-aryl ether lignin model compound, Kraft lignin and wheat straw lignocellulose [6,13,14]. The significant progress in understanding the catalytic mechanisms, crystal structures, and expression regulations of some of the microbial lignin-degrading enzymes is highlighted in a couple of recent reviews [15,16].

Thermobifida fusca, a moderately thermophilic filamentous bacterium commonly found in decaying organic matter, has long been known to secrete various cellulolytic enzymes in media containing cellulosic materials [17]. Genome analysis confirmed that it is capable of producing an array of cellulases, including endocellulase, exocellulase, cellobiosidase, and β-glucosidase, and at least 5 hemicellulose hydrolysis-related enzymes [18]. Although this bacterial genome lacks fungal laccase-like protein-encoding gene, open reading frame 1114 encodes a monocopper oxidase (designated as Tfu1114) that exhibits activity for the oxidation of 2,6-dimethoxyphenol (2,6-DMP), a common substrate for laccase activity, and veratryl alcohol [19]. Interestingly, Tfu1114 enhances the capability of the bacterium's own xylanase/cellulase to release reducing sugars from sugarcane bagasse. This study has investigated the underlying mechanism enabling Tfu1114 to assist cellulolytic enzymes for bagasse hydrolysis and further explored the potential of Tfu1114 in the development of an environment friendly process for lignin removal.

2. Materials and methods

2.1. Materials

Alkaline (Kraft) lignin (CAS #8068-05-1) was purchased from Sigma-Aldrich. Sugarcane bagasse was collected from the local market and washed extensively with tap water to remove residual soluble sugars. Then, it was dried at 50 °C for 2 days and smashed into powder with sizes smaller than 100 mesh. To determine whether Tfu1114 is able to modify the lignin structure in bagasse, 10 g of the powdered bagasse was pretreated with 1 g *Aspergillus niger* cellulase (Sigma-Aldrich, C1184, ≥300 U/g) and 100 U of *T. fusca* xylanase [20] in a final volume of 100 ml Tris buffer (20 mM, pH 7.0) at 50 °C for 4 days. The reaction mixture was supplemented with 0.2% sodium dodecyl sulfate (SDS) and heated in boiling water for 20 min to inactivate the enzymes. The pretreated bagasse was collected using glass wool filters, washed with water, and dried at 50 °C.

Cellulases of three origins were used in combination with Tfu1114 in bagasse hydrolysis experiments. *Aspergillus* sp. cellulase was purchased from Sigma-Aldrich (C2605, 1200 U/ml). Cellulases prepared from the broth of *Trichoderma reesei* (600 U/g) and *Acremonium cellulolyticus* (1000 U/g) were gifts from Challenge Bioproducts Company (Touliu, Taiwan).

2.2. Methods

2.2.1. Protein expression and purification

Tfu1114 expression in recombinant *E. coli* BL21(DE3) was as described previously [19]. Cell pellets harvested from 0.5 l culture broth were suspended in 50 ml 20 mM *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) [pH 8.0] and vigorously agitated with a vortex (Vortex-Genie 2, Scientific Industries, USA) at the maximal rate for 2 min. The supernatant after centrifugation at 10,000 rpm for 20 min was saved as the crude enzyme preparation. The crude Tfu1114 was purified by immobilized metal affinity chromatography using a 10 ml nickel-nitrilotriacetic acid (Ni-NTA) column. Tfu1114 bound to the column was finally eluted with buffer that contained 20 mM TAPS [pH 8.0], 100 mM NaCl, 50 mM KCl, 10% (v/v) glycerol, and 500 mM imidazole. To prepare the purified Tfu1114 for experiments involving mass spectrometry, 20 mM ammonium acetate [pH 8.0] was substituted for TAPS buffer. Elution fractions that contained Tfu1114 were dialyzed against a 100-fold volume of ammonium acetate [pH 8.0] in the presence of 0.1% SDS at 4 °C overnight. Dialysis was performed 3 times to remove imidazole as completely as possible.

2.2.2. Composition analysis of lignocellulose

The amounts of cellulose, hemicellulose, and lignin in sugarcane bagasse were quantified based on the methods developed by van Soest et al. [21]

2.2.3. Oxidation activity assay

The activity of Tfu1114 was monitored by incubating the enzyme with 2 mM 2,6-DMP in 20 mM TAPS buffer [pH 8.0] plus 100 mM NaCl at 50 °C. The activity was calculated based on the increase in absorbance at 470 nm ($\varepsilon = 35645\ M^{-1}\ cm^{-1}$). One unit was defined as the activity to produce 1 μmol of the oxidized product of 2,6-DMP per minute.

2.3. Hydrogen peroxide production assay

The oxidation of 2,6-DMP (2 mM) by Tfu1114 (5 mU/ml) was performed in 20 mM TAPS buffer [pH 8.0] plus 100 mM NaCl at 50 °C for 10 min. Alkaline lignin (1 mg/ml) and bagasse (10 mg/ml) was oxidized by Tfu1114 (50 mU/ml) under the same reaction condition for 8 h. The reaction mixture was aerated with oxygen at a rate of 2 gas volumes per liquid volume per minute (vvm). Hydrogen peroxide accumulated at the indicated time period was measured using OxiVision Green™ hydrogen peroxide sensor (AAT Bioquest, Sunnyvale, CA), which exhibits a > 100-fold selectivity for hydrogen peroxide over other reactive oxygen species. The sample was mixed with an equal volume of freshly prepared 10 μM OxiVision Green™ hydrogen peroxide sensor solution, and the mixture was placed onto a 96-well black plate and incubated at room temperature in the dark for 60 min. Increase of the fluorescence at Ex/Em = 485/528 nm was measured using a microplate reader Synergy H1 (BioTek Instruments, Winooski, VT). Hydrogen peroxide concentration was calculated according to the standard curve.

2.4. Alkaline lignin modification assay

The reaction solution (5 ml) contained 0.25 U of the purified Tfu1114, 5 mg alkaline lignin, 20 mM TAPS buffer [pH 8.0], and 150 mM NaCl. The reaction was performed at 50 °C for 96 h using a glass tube with a ventilation stopper through which oxygen gas was pumped into the solution at an aeration rate of 2 vvm. The absorbance of the reaction products was recorded over the wavelength range of 200–800 nm using a VIS-UV spectrophotometer. The total phenolic content was determined by Folin-Ciocalteu

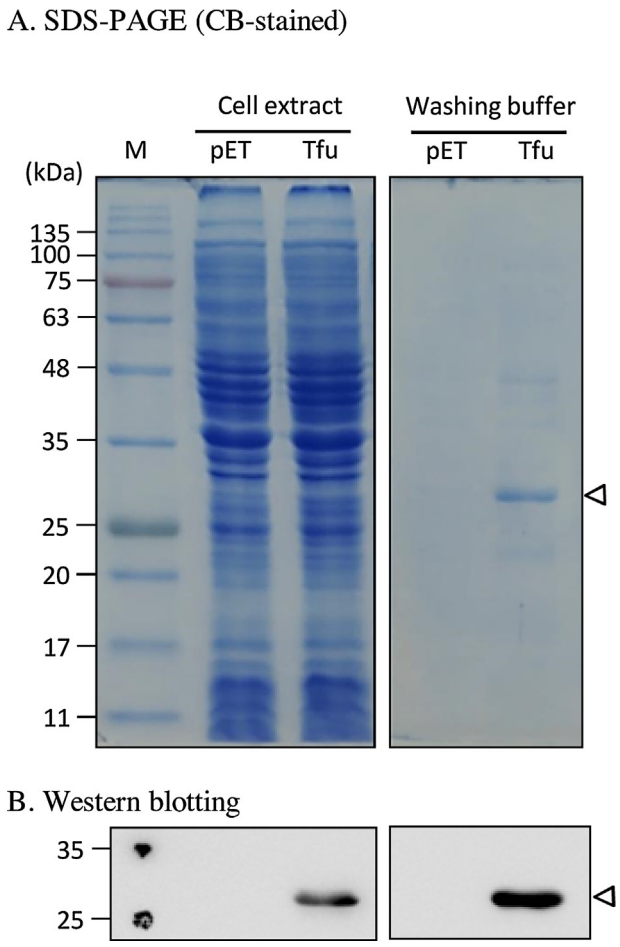

Fig. 1. Release of the recombinant Tfu1114 from *E. coli* by vigorous vortexing. pET and Tfu denote the samples from *E. coli* BL21(DE3) cells that harbored pETDuet and pETDuet-Tfu1114 respectively. Washing buffer denotes the used buffer that had been in vigorous agitation with the recombinant cells. Proteins in the indicated samples were analyzed by SDS-12% PAGE (*A*) and western blotting using anti-Tfu1114 antiserum (*B*).

reagent [22], and the molecular weight distribution was examined by high-performance size-exclusion chromatography (HPSEC). The column used was TSKgel G2000SWXL (7.8 mm × 30 cm, 0.5 μM particles) (Tosoh, Japan), and the mobile phase was 20 mM TAPS buffer [pH 8.0] plus 100 mM NaCl at a flow rate of 0.75 ml/min.

2.5. Modification of pretreated sugarcane bagasse by Tfu1114

The reaction was performed by incubating 0.1 g pretreated sugarcane bagasse with 1 U of the purified Tfu1114 in 10 ml solution that also contained 20 mM ammonium acetate [pH 8.0] and 100 μM $CuSO_4$ at 50 °C, supplied with oxygen gas at 1 vvm. The used dialysis buffer in Tfu1114 preparation served as a control to treat the bagasse. The oxidation of bagasse was continued for 5 days by replenishing Tfu1114 (1 U) every 24 h. Finally, the mixture was supplied with 0.2% SDS and heated in boiling water for 20 min to denature Tfu1114. The Tfu1114-treated bagasse debris was collected using glass wool filters, washed extensively with distilled water, and dried at 50 °C. The supernatant withdrawn on day one was saved for mass spectrometric assay, while the dried bagasse debris was used in Fourier transform infrared (FTIR) analysis.

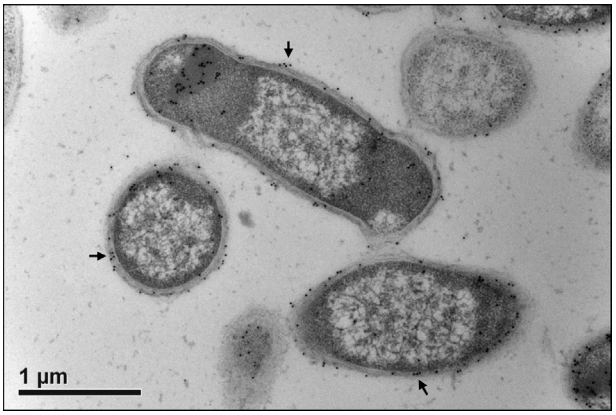

Fig. 2. Subcellular imaging of Tfu1114. *E. coli* cells that expressed Tfu1114 were labeled with anti-Tfu1114 antibodies and colloidal gold-conjugated secondary antibodies and viewed under a transmission electron microscope. The arrows point out some Tfu1114 molecules that had been recognized by the antibodies and presented as dots.

2.6. Mass spectrometric assay

The sample was acidified to contain 5% acetic acid prior to liquid chromatography. The Hypersil Gold C_{18} column (100 mm × 2.1 mm, 5 mm, Thermo Fisher Scientific, MA, USA) was operated on the Dionex Ultimate 3000 system (Gemering, Germany). The elution program was: 20–30% methanol/water, 0–5 min; 30–50% methanol/water, 5–12 min; 50–80% methanol/water, 12–30 min; at a flow rate of 0.2 ml/min. The analytes were ionized by removing a proton using an electrospray probe and detected using HCT ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) under the conditions: nebulizer gas, 30 psi; drying gas, 8 l/min; drying temperature, 300 °C; capillary voltage, 3.5 kV; scanning range, 100–1000 *m/z*. All the data was analyzed using the program Data analysis version 4.0 (Bruker).

2.7. FTIR analysis

The Tfu1114-treated bagasse was examined at room temperature with an attenuated total reflection FTIR (Spectrum 100, Perkin–Elmer, Bucks, UK), equipped with a deuterated triglycine sulfate detector and a MIRacle ATR accessory (Pike Technologies, WI, USA). The scan was in the range from 4000 to 650 cm^{-1} at a resolution of 4 cm^{-1}. The spectrum of each sample was an average of 32 scans.

2.8. Transmission electron microscopy (TEM)

Tfu1114-expressing *E. coli* cells were fixed with 2.5% glutaraldehyde, dehydrated, and embedded into L.R. White resin (Polysciences, Warrington, PA). Tfu1114 in sliced sections, 60-nm thickness, was labeled by mouse anti-Tfu1114 antiserum, followed by 15 nm gold-conjugated goat anti-mouse IgG antisera (British Biocell International, UK). The immune-labeled sections were then treated with 2% uranyl acetate for 20 min and 0.5% lead citrate for 10 min and observed using the JEM-1400 transmission electron microscope (JEOL, Peabody, MA).

2.9. E. coli cultivation using bagasse hydrolysate

50 mg dried sugarcane bagasse, 0.5 ml the crude preparation of Tfu1114 (0.22 U/ml) and indicated amounts of fungal cellulases were mixed in final 5 ml 20 mM TAPS buffer [pH 8.0] in a 50 ml conical tube. The tube, with the screw cap loosely closed, was placed on a rotary shaker, 200 rpm, and incubated at 50 °C for 2 days. Sam-

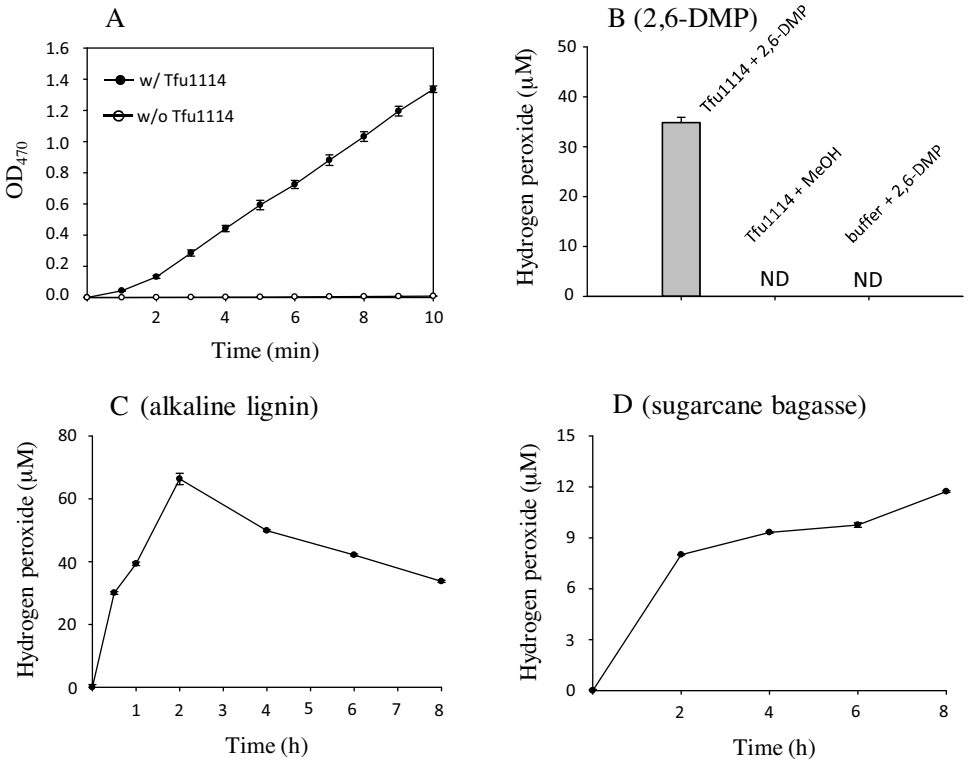

Fig. 3. Hydrogen peroxide production in reactions catalyzed by Tfu1114. The reactions were performed at 50 °C as described in Materials and methods, and the accumulated concentration of hydrogen peroxide was quantified by OxiVision Green™ solution. (*A*), reaction of 2,6-DMP with (w/) or without (w/o) Tfu1114 over a 10 min period. Changes in OD_{470} nm were recorded. The data are averages of three experiments. (*B*), the hydrogen peroxide concentration at the 5-min reaction as described in (*A*) was determined. ND denotes an undetectable amount of hydrogen peroxide. (*C*), alkaline lignin was oxidized by Tfu1114 and the hydrogen peroxide accumulated with time was determined. (*D*), sugarcane bagasse was oxidized by Tfu1114 and the hydrogen peroxide accumulated with time was determined.

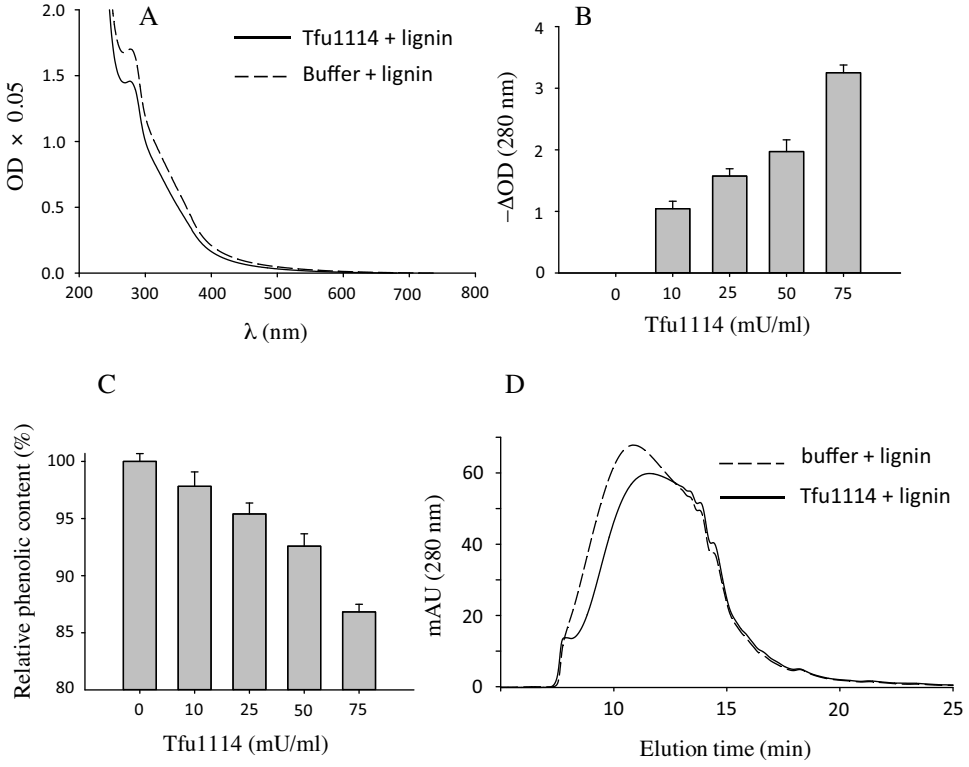

Fig. 4. Modification of alkaline lignin by Tfu1114. The reaction conditions were as described in Materials and methods. (*A*), the lignin was treated with 50 mU/ml of Tfu1114 or buffer (as a control) and the products were scanned from 200 to 800 nm using a spectrophotometer. (*B*), the lignin was treated with Tfu1114 and the change of absorbance at 280 nm was plotted versus the indicated amounts of Tfu1114. (*C*), the lignin was treated with Tfu1114 and the change in the relatively total phenolic content was plotted versus the indicated amount of Tfu1114. (*D*), 50 mU/ml of Tfu1114 was used in the reaction and the products were analyzed by high-performance size-exclusion chromatography using the TSKgel G2000SWXL column. Buffer instead of Tfu1114 was used in the control.

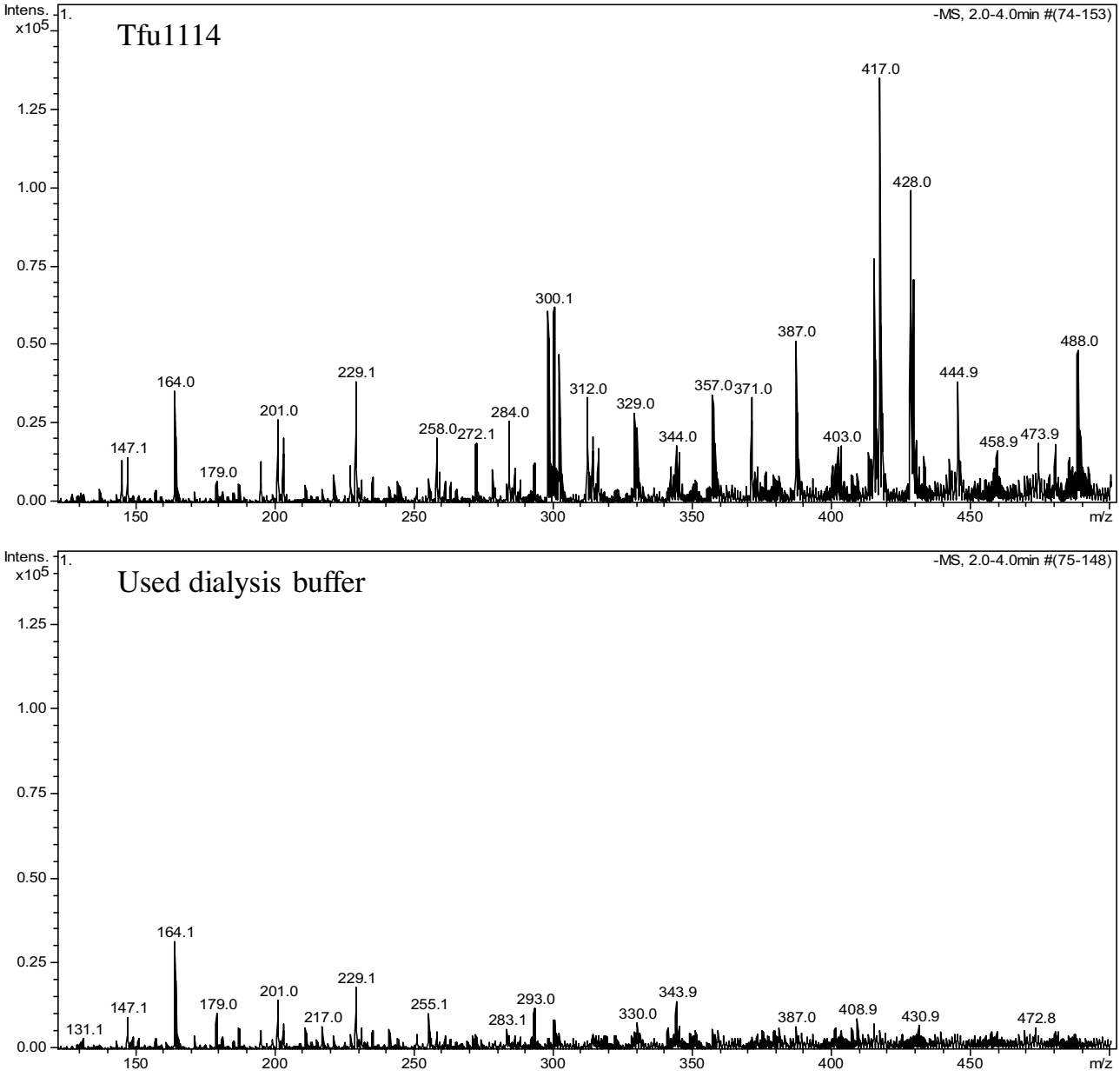

Fig. 5. Analysis of the species released from bagasse by LC–MS spectrometry. The pretreated sugarcane bagasse was incubated with Tfu1114 or the used dialysis buffer as described in Materials and methods. The supernatant of the samples was analyzed by LC–MS spectrometry. The intensity versus *m*/*z* value of the species eluted within the retention time from 2 to 4 min is presented.

ples at the indicated intervals were assayed for the accumulated amount of reducing sugars by dinitrosalicylic acid method using glucose as the standard. To test the usability of the hydrolysate of bagasse for growth of microorganisms, the hydrolysate was used as the basal solution to prepare R/2 minimal medium [23]. After sterilization by filtration through a 0.22-μm filter, the medium was used to cultivate *E. coli* BL21(DE3) cells. The bacterial growth in R/2 media containing the indicated amount of glucose or equivalent reducing sugars from the hydrolysate was monitored using a microplate reader.

3. Results

3.1. *Tfu1114 is a secreted protein in* E. coli

In previous experiments, we noticed that the yield of Tfu1114 decreased drastically if the recombinant *E. coli* cells had been washed extensively before disruption by regular means such as sonication. To reason this observation, we analyzed the used buffer that had been in agitation with the cells for the presence of Tfu1114. Analyses of the samples by SDS-PAGE and western blotting using anti-Tfu1114 antiserum showed the presence of Tfu1114 in the used buffer (Fig. 1). It is worth noting that Tfu1114 in the used buffer was abundant compared to other proteins. The enrichment specific for Tfu1114 suggests that the proteins in the buffer were not from the cell lysate. In addition, Tfu1114 was indiscernible in the culture medium (data not shown). Together, these results imply that Tfu1114 was secreted across the cytoplasmic membrane and anchored on the outer surface of the cells, from which the protein could be dislodged by vigorous vortexes. To provide conclusive evidence for the location of Tfu1114 on the bacterial surface, the Tfu1114-expressing *E. coli* cells were labeled using anti-Tfu1114 antiserum and the colloidal gold-conjugated secondary antiserum prior to TEM observation. The TEM picture clearly indi-

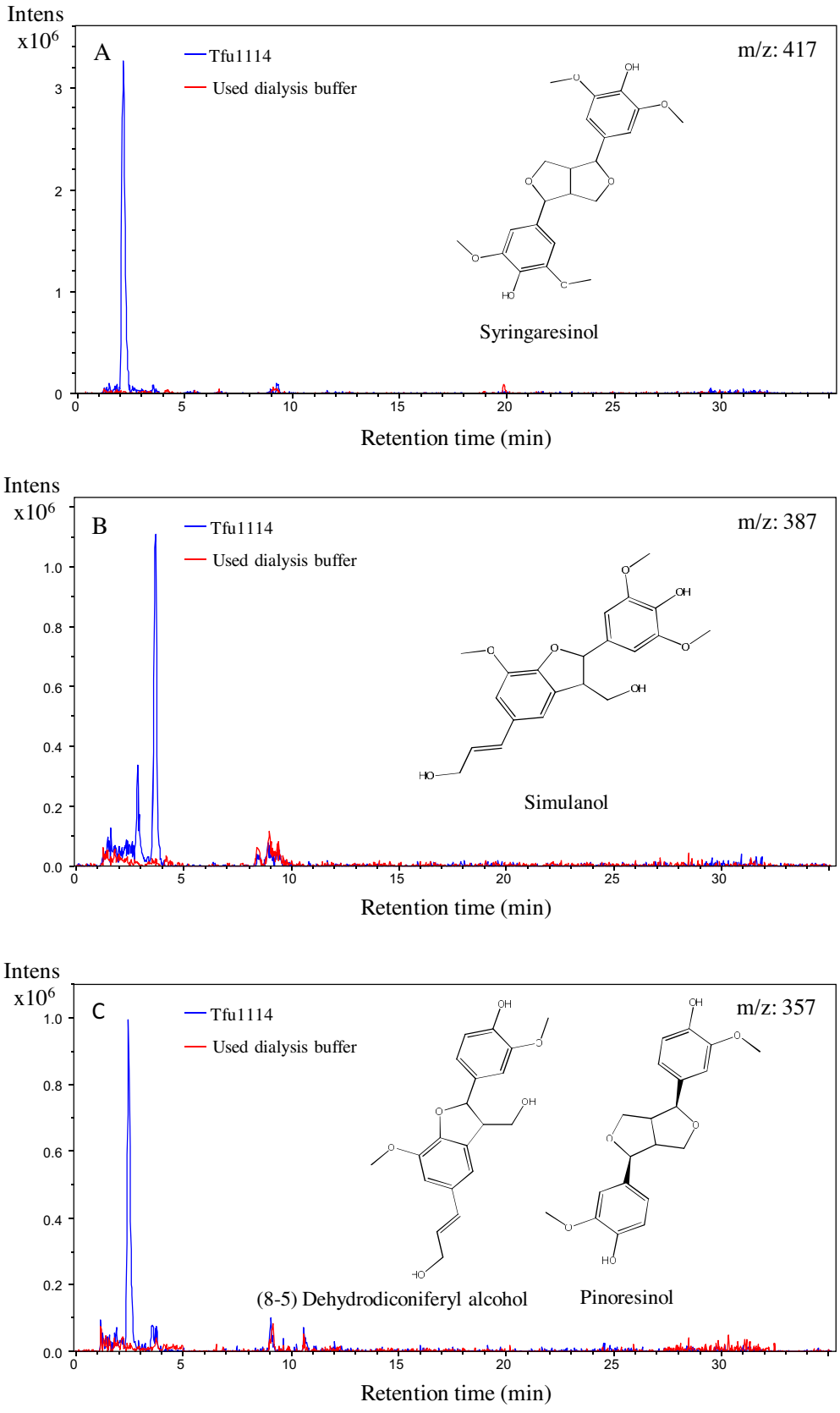

Fig. 6. Elution profile of selective species released from the Tfu1114-treated bagasse. The pretreated sugarcane bagasse was incubated with Tfu1114 or the used dialysis buffer as described in Materials and methods. The supernatant of the samples was analyzed by LC–MS spectrometry. The elution profiles of species with *m*/*z* of 417 (*A*), 387 (*B*), and 357 (*C*) are presented. The proposed molecules based on the values of *m*/*z* are depicted.

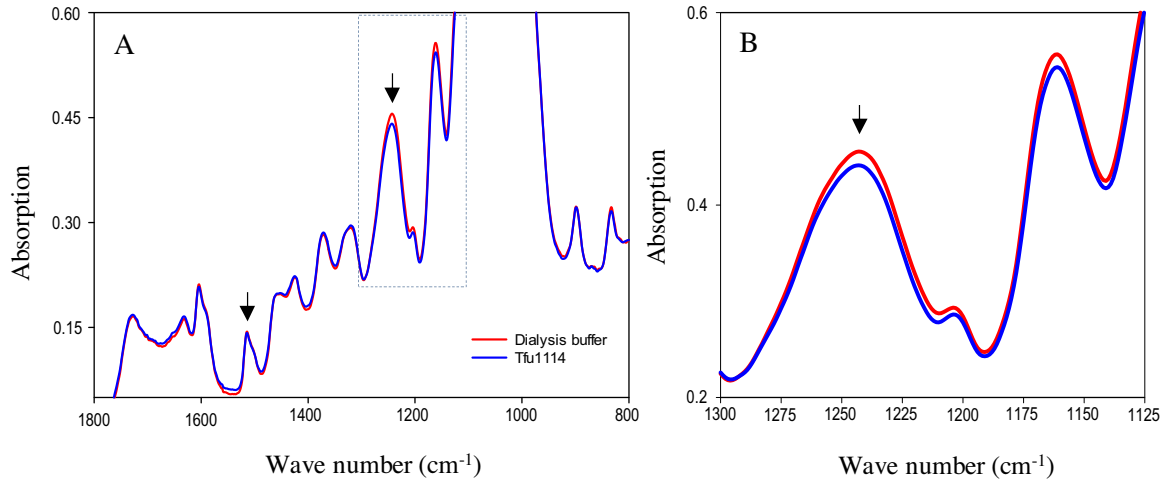

Fig. 7. Changes in infrared spectra of bagasse after the treatment with Tfu1114. The pretreated sugarcane bagasse was incubated with Tfu1114 or the used protein dialysis buffer as described in Materials and methods. The debris was analyzed by FTIR spectrometry. (*A*), the spectra over the wave number 800–1800 cm^{-1} are presented. The two typical absorption bands of lignin are indicated by arrows. (*B*), the spectra from 1125 to 1300 cm^{-1} are selectively presented.

cates that the majority of Tfu1114 molecules resided on the cell envelope, particularly the outer membrane (Fig. 2). Obviously, Tfu1114 was secreted by *E. coli* although it lacks a typical *sec*-dependent signal peptide or twin arginine translocation signal at the N terminus.

3.2. *Tfu1114 is a hydrogen peroxide-generating oxidase*

Tfu1114 is able to oxidize several phenolic and nonphenolic aromatic compounds, presumably by using molecular oxygen as the final electron acceptor; nonetheless, whether the reduction product of oxygen is water or hydrogen peroxide was undetermined [14]. To find the answer, the oxidation of 2,6-DMP (2 mM) by Tfu1114 was monitored over a 10 min period and the presence of hydrogen peroxide in the solution was determined. The steadily increased absorbance at 470 nm over the period suggests that the oxidized product of 2,6-DMP, probably a biphenyl dimer, accumulated at an approximate rate of 7 μM/min under this reaction condition (Fig. 3A). Approximately 36 μM hydrogen peroxide was detected in the 5-min reaction solution; by contrast, no hydrogen peroxide was detected in reactions without Tfu1114 or 2,6-DMP (Fig. 3B). This results indicate a 1:1 ratio of the oxidized product and hydrogen peroxide; in other words, the electrons withdrawn from 2,6-DMP are completely used to reduce oxygen to hydrogen peroxide. Furthermore, Tfu1114 was incubated with alkaline lignin and sugarcane bagasse, and hydrogen peroxide in the reaction mixture was measured. Hydrogen peroxide accumulated rapidly in the first 2 h and reached a peak of ~67 μM in the reaction that contained 1 mg/ml alkaline lignin and 50 mU/ml of Tfu1114 (Fig. 3C). Hydrogen peroxide rapidly accumulated in the initial phase might inactivate the activity of Tfu1114 through oxidations of active-site amino acid residues; therefore, the steady state level of hydrogen peroxide would decline gradually after the peak. As to sugarcane bagasse (10 mg/ml), hydrogen peroxide was increased steadily and reached ~12 μM over an 8-h reaction period (Fig. 3D). The insoluble nature of bagasse could be the primary factor rendering bagasse a less favorable substrate than alkaline lignin in terms of oxidation by Tfu1114. Taken together, Tfu1114 is a hydrogen peroxide-generating oxidase, distinct from laccase that produce water coupled to the substrate oxidation.

3.3. *Tfu1114 reduces the phenolic content of alkaline lignin*

Changes in the physicochemical properties of alkaline lignin after oxidation by Tfu1114 were further examined. The absorbance spectrum of alkaline lignin after a 96-h treatment by Tfu1114 was scanned from 200 to 800 nm. Tfu1114 decreased the absorbance over the entire scanned range compared with that treated with buffer (Fig. 4A). The maximal change occurred at ~280 nm, and the magnitude of the change increased with the increased amount of Tfu1114 (Fig. 4B). Although alkaline lignin has been used as a model compound for a long time, it actually has more phenol constituents than natural lignin. The total phenolic content of this substrate decreased after the treatment with Tfu1114; in addition, Tfu1114 exhibited a dosage effect on the reduction of the phenolic content (Fig. 4C). Specifically, the decreasing rate of total phenolic content of 1 mg alkaline lignin in percentage (%) was about 0.45 per 10 mU Tfu1114 per day. To know whether the molecular weight distribution of species in alkaline lignin was altered by Tfu1114, the substrate treated with Tfu1114 was analyzed by size-exclusion chromatography using the TSKgel G2000SWXL column (Fig. 4D). The profiles of absorbance at 280 nm versus elution time confirm that alkaline lignin was a highly heterogeneous mixture composed of species with a wide range of molecular weights. The treatment with Tfu1114 reduced the height of the peak by ~17% in the elution region corresponding to the species with higher molecular weights.

3.4. *Tfu1114 modifies the lignin structure in bagasse*

It was important to determine whether Tfu1114 is also able to attack natural lignin. For this specific aim, sugarcane bagasse was pretreated with *Aspergillus* cellulase and *Thermobifida* xylanase as described in Materials and methods, hoping to render the lignin in the bagasse more accessible to Tfu1114. The composition of 1 g raw bagasse was determined to contain 458 mg cellulose, 236 mg hemicellulose, and 191 mg lignin. A slight change was made by the enzymatic treatment in that 1 g sample had 447 mg cellulose, 228 mg hemicellulose, and 202 mg lignin. The pretreated bagasse was incubated with Tfu1114, and the supernatant of the reaction mixture was analyzed by LC–MS spectrometry to identify possible phenolic derivatives arising from lignin degradation. A great difference in signals was found between the Tfu1114-treated sample and the control within the retention times of 2–4 min (Fig. 5). Many species such as those with *m*/*z* of 417 and 428 appeared

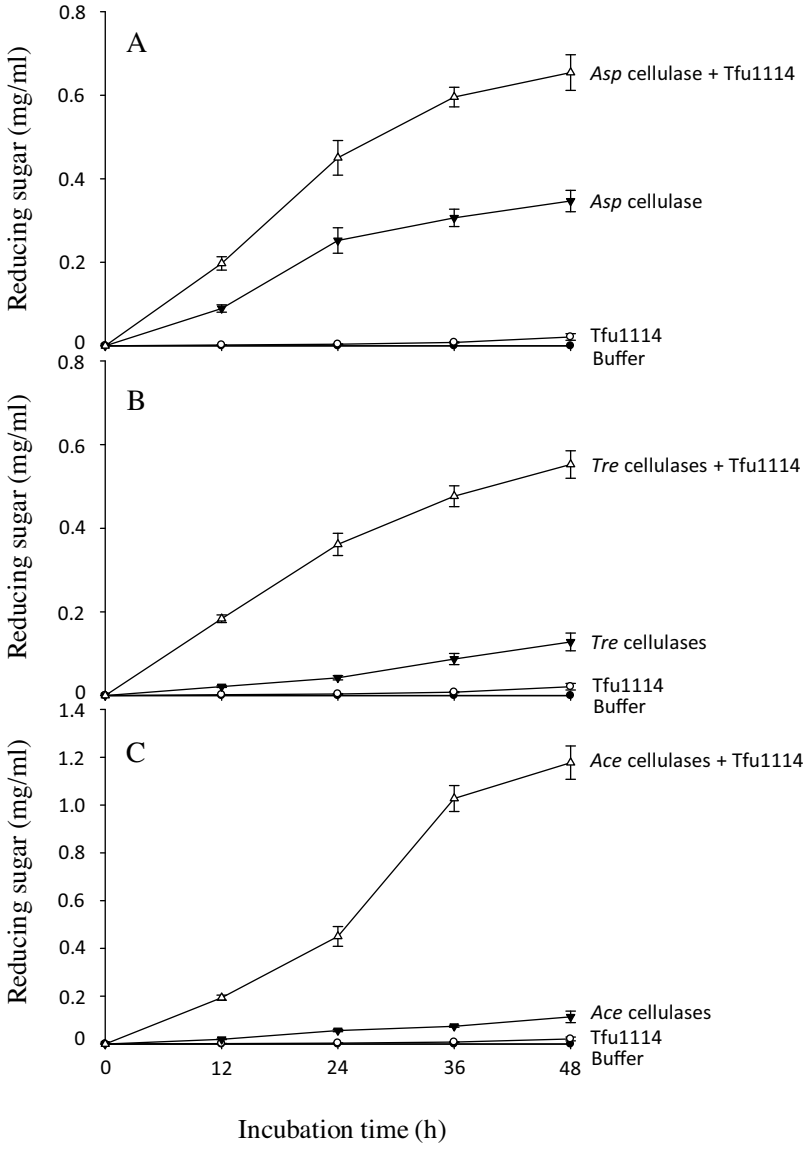

Fig. 8. Accumulation of reducing sugars in the enzymatic hydrolysate of sugarcane bagasse. The reaction condition was as described in Materials and methods. The amount of *Aspergillus* cellulases (*Asp*, panel A) was 240 U, while the amount of cellulases of *Trichoderma reesei* (*Tre*, panel *B*) and of *Acremonium cellulolyticus* (*Ace*, panel *C*) was 5 U. 110 mU of Tfu1114 was used when it was needed. The data are averages of three independent experiments.

only in the Tfu1114-treated sample. The elution profiles of some of the representative species are shown in Fig. 6. According to the agreement of the molecular mass, the species with m/z of 417 is proposed to be syringaresinol, a dilignol coupled by two molecules of sinapyl alcohol via the C8—C8′ bond (Fig. 6A). The species with m/z of 387 probably is simulanol, formed by connecting the C5 of a coniferyl alcohol to the C8′ of a sinapyl alcohol (Fig. 6B). The species with m/z of 357 may represent either dehydrodiconiferyl alcohol or pinoresinol, linked by two coniferyl alcohols via a C5—C8′ or C8—C8′ bond, respectively (Fig. 6C). Production of these dilignols, common constituents of lignin, supports the notion that Tfu1114 is capable of breaking some linkages of lignin in sugarcane bagasse. Peroxides have been known for their activity to modify lignin and lignin model compounds [24]; therefore, the pretreated bagasse was also treated with 180 μM H_2O_2 at the same condition as with Tfu1114 to know if H_2O_2 itself is sufficient to release lignols from the bagasse. According to the LC–MS analysis, no degradation product was detected in the H_2O_2-treated sample in significant amounts compared to that treated with Tfu1114 (data not shown). We guess that peroxides must be close to the target bonds to break the bonds because they are short lived, particularly at 50 °C.

To confirm Tfu1114 being a ligninolytic enzyme, the Tfu1114-treated bagasse was examined by FTIR spectrometry. Lignin exhibits two distinctive absorption bands under infrared: (i) the aromatic ν(C=C) (semicircle ring stretch), 1530–1480 cm^{-1}; and (ii) the broader combined ν(C—C) & ν(C—O) bands, 1260–1210 cm^{-1} [25,26]. The FTIR spectrum of the pretreated bagasse contained the two typical bands of lignin along with others (Fig. 7). Oxidation of the bagasse by Tfu1114 did not change the absorption band between1530–1480 cm^{-1} but reduced the intensities of absorption at 1240, 1210, and 1160 cm^{-1}. Accordingly, Tfu1114 was devoid of the activity to break the double bond of aromatic rings; nonetheless, it appeared to reduce the number of ether and/or alkyl bonds adjacent to aromatic rings. To cellulosic materials, the absorption band at 1160 cm^{-1} may represent the C—H in-plane deformation of the guaiacyl ring (derived from coniferyl alcohol) and C—O—C asymmetric stretching in cellulose [27]. Because Tfu1114 was the determinant factor causing the spectral changes, the intensity

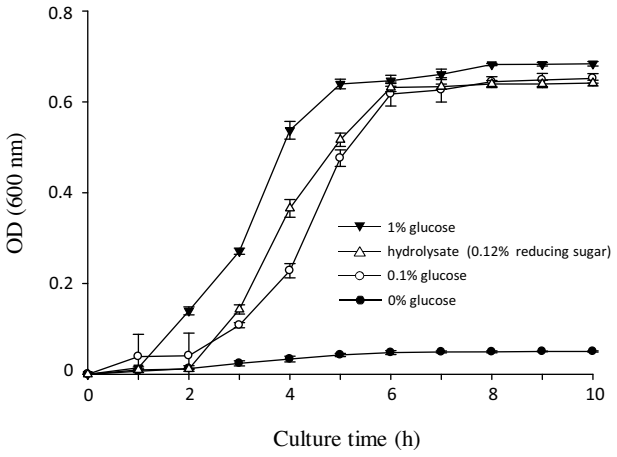

Fig. 9. Time courses of *E. coli* grown in R/2-based minimal media. The overnight culture of *E. coli* BL21(DE3) in LB medium was washed three times with glucose-free R/2 medium and transferred (1:100) into R/2 minimal media that also contained the indicated amounts of glucose or reducing sugar from bagasse hydrolysate. At the indicated intervals, 200 μl of the broth was transferred into a well of the 96-well plate and the optical density at 600 nm of the broth was measured using a microplate reader.

decrease at 1160 cm^{-1} was more likely due to changes in the C—H in-plane deformation of the guaiacyl ring.

3.5. *Tfu1114 help to produce usable sugars from lignocellulose*

Although Tfu1114 was known to enhance the hydrolyzing activity of *T. fusca* xylanase/cellulase toward bagasse, whether it is workable with commercial fungal cellulases was tested in this study. Sugarcane bagasse (10 mg/ml) was incubated at 50 °C with cellulases prepared from *Aspergillus* sp., *Trichoderma reesei*, or *Acremonium cellulolyticus*, along with or without Tfu1114 for 2 days. The crude preparation of Tfu1114 was used in this specific study to see if the cost for further enzyme purification can be saved. The time courses showed that the reducing sugar accumulated more and faster in the reactions that simultaneously contained cellulases and Tfu1114 (Fig. 8). Reducing sugars in the solution containing *Acremonium* cellulases and Tfu1114 reached 1.2 mg/ml at 48 h, a 10-fold increase compared with that treated with the fungal cellulases only (Fig. 8C). This production corresponds to a yield of about 26% under the assumption that all the reducing sugars came from cellulose in bagasse. Pretreatment of lignocellulose for the removal of lignin by acids are prone to produce toxic compounds such as furfural and 5-hydroxymethylfurfural [28,29], which impede the growth of microorganisms. To know if the reducing sugars obtained from the above preparation are usable, the bagasse hydrolysate was directly included in R/2-based minimal medium to cultivate *E. coli* cells (Fig. 9). The cells grown in the 1% glucose-containing minimal medium started to grow exponentially at 1 h post-inoculation, while those in the medium containing 0.1% glucose or 0.12% reducing sugar equivalents (from bagasse hydrolysate) started at 2 h post-inoculation. Nonetheless, the cells in all the three media exhibited similar specific growth rates at the exponential phase and reached plateaus at about 0.60–0.63 OD. This result suggests that the hydrolysate did not contain significant amounts of toxic chemicals that commonly produced in the lignin-removing processes using liquid hot water and dilute acids.

4. Discussion

This study unexpectedly found that Tfu1114 was secreted across the cytoplasmic membrane in *E. coli* despite the lack of *sec*-dependent and twin arginine translocation signals. The recombinant Tfu1114 recovered from the agitation buffer was able to bind to Ni-NTA resin, suggesting that the engineered polyhistidine tag was still at the N terminus of the recombinant protein. Given the fact that the authentic Tfu1114 was present in the culture medium of *T. fusca* [19], these two bacteria may share a common unidentified mechanism for the secretion of Tfu1114. Finding the secretion machinery and cargo proteins that can be secreted by this mechanism may be academically meaningful. From the viewpoint of application, the ease of isolating the recombinant Tfu1114 from *E. coli* cells by merely vigorous agitation for a short period of time may represent an advantage of using this enzyme in the biotechnologically relevant processes.

Polyphenol oxidases are a widespread group of enzymes found in plants, fungi and bacteria. Laccases, a group of multicopper oxidase, catalyze the oxidation of a variety of phenolic and nonphenolic lignin related compounds coupled to the reduction of molecular oxygen to water. With the oxidation activity, laccases have been considered excellent candidates for many industrial and biotechnological applications, such as decolorization of textile dyes and bioremediation of polluted soils and water [30,31]. As for enhancing the efficiency of cellulases in the hydrolysis of lignocellulosic biomass, the laccase isolated from *Cerrena unicolor* was shown recently to improve the enzymatic hydrolysis of steam-treated spruce (*Picea abies*) by 12% [32]. Tfu1114, a monocopper oxidase, also oxidizes some phenolic and nonphenolic compounds; however, the electrons taken away from the substrates reduce oxygen to hydrogen peroxide. Besides, Tfu1114 is resistant to sodium azide [19], a potent inhibitor of laccases. These fundamental differences indicate a distinct catalytic mechanism employed by Tfu1114. This study also demonstrates that Tfu1114 is a lignin-modifying enzyme able to attack the lignin in sugarcane bagasse probably by breaking some C—C and C—O bonds adjacent to aromatic rings, leading to the production of hydroxycinnamyl alcohol-derived compounds such as syringaresinol and pinoresinol. Moreover, it is important to confirm that Tfu1114 could help commercial cellulases to release reducing sugars from bagasse by up to several folds.

Production of hydrogen peroxide coupled to oxidation of lignin may be important to the activity enhancement of cellulolytic enzymes by Tfu1114. Hydrogen peroxide may enter the Fenton reaction to produce free hydroxyl radicals, which immediately break the nearby lignol-connecting bonds. The cleavages created by Tfu1114 may facilitate the penetration of lignocellulose by cellulolytic enzymes. In compost, hydrogen peroxide may be used as a co-substrate by peroxidases secreted from *T. fusca* or other microbes to continue the degradation of lignin. Actually, a DyP-type peroxidase from *T. fusca* was reported to decolorize a variety of aromatic dyes [33]. It will be interesting to determine the effect of the DyP-type peroxidase on cellulase-catalyzed lignocellulose hydrolysis, particularly when Tfu1114 is present.

Because of the wealth of genetic and metabolic knowledge associated with *E. coli*, the metabolic pathways of this bacterium has been engineered in a variety of ways in order to convert renewable sugars into biofuels such as ethanol and other short-chain alcohols [34]. One of the bottlenecks needed to be overcome to optimize this production process is to reduce furfural and furan production in the chemical processing step of lignocellulosic biomass or to enhance the resistance of *E. coli* to these toxins. This study used bagasse hydrolysate-based R/2 medium to cultivate *E. coli* BL21 strain and demonstrated that the medium was as effective as the regular glucose-containing R/2 medium for supporting the growth of *E. coli* cells. Given the fact that *E. coli* is sensitive to furfural, furan and their chemical derivatives, the comparable growths of *E. coli* in both media suggest that the toxins were absent, or at ignorable concentrations, in the hydrolysate. From an applied perspective,

Tfu1114, therefore, has potential to provide an eco-friendly way to modify lignin so that not only the access of cellulolytic enzymes to the polysaccharides embedded in lignocellulose is increased but also the generation of toxic compounds due to the pretreatment of lignocelluloses by chemicals can be avoided.

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