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Development of a novel cellulase biosensor that detects crystalline cellulose hydrolysis using a transcriptional regulator

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ABSTRACT

Successful utilization of cellulose as renewable biomass depends on the development of economically feasible technologies, which can aid in enzymatic hydrolysis. In this study, we developed a whole-cell biosensor for detecting cellulolytic activity that relies on the recognition of cellobiose using the transcriptional factor CelR from *Thermobifida fusca* and transcriptional activation of its downstream *gfp* reporter gene. The fluorescence intensity of whole-cell biosensor, which was named as cellobiose-detectible genetic enzyme screening system (CBGESS), was directly proportional to the concentration of cellobiose. The strong fluorescence intensity of CBGESS demonstrated the ability to detect cellulolytic activity with two cellulosic substrates, carboxymethyl cellulose and *p*-nitrophenyl β-D-cellobioside in cellulase-expressing *Escherichia coli*. In addition, CBGESS easily sensed crystalline cellulolytic activity when commercial Celluclast 1.5L was dropped on an Avicel plate. Therefore, CBGESS is a powerful tool for detecting cellulolytic activity with high sensitivity in the presence of soluble or insoluble cellulosic substrates. CBGESS may be further applied to excavate novel cellulases or microbes from both genetic libraries and various environments.

1. Introduction

A genetic circuit-based biosensor is able to detect specific small molecules produced from a desired biological pathway or an enzyme reaction [1–3]. In general, the genetic circuit relies on a quantifiable phenotype detected by a genetically encoded reporter such as fluorescent proteins. The small molecule in metabolic engineering originates from multiple steps of cellular metabolism, whereas enzyme engineering employs one- or few-step enzyme reactions. Transcriptional regulator-based biosensors convert products of enzymatic reactions to measurable signals using specific interaction between transcriptional regulator and its effectors. A screening system using a biosensor has several advantages including extensive expression, linkages between genotype (coding enzymes) and phenotype (expressing reporter genes) and high-throughput screening in combination with flow cytometry.

Cellulose is the most abundant renewable and low-cost biological resource. It is a glucose polymer linked by a β-1,4-glycosidic bond, which can be hydrolyzed in a synergetic cellulolytic enzyme reaction involving endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) [4,5]. Endoglucanase randomly hydrolyzes accessible intramolecular β-1,4-glycosidic bonds of cellulose to yield oligosaccharides. Simultaneously, exoglucanase degrades crystalline cellulose to soluble cellobiose and then β-1,4-glucosidase hydrolyzes cellobiose to glucose to be metabolized into valuable products. Despite the abundance of cellulose, the availability of valuable products produced from cellulose is limited by the difficulty of cellulose hydrolysis, which is primarily related to structural recalcitrance of cellulose [6,7].

To develop enzymes for the efficient hydrolysis of cellulose, powerful and robust screening techniques are required to identify and engineer novel cellulases. Conventional methods for cellulase screening based on microtiter plates or solid media are labor-intensive and time-consuming [8,9]. Moreover, these methods exhibit limited use for mining processive cellulases in terms of

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hydrolysis of crystalline cellulose. Although a few detection techniques were developed for exocellulase degrading crystalline cellulose, all of them utilized specific unnatural substrates that may cause ambiguous results in the initial screening steps [10,11].

Previously we presented the utility of the Genetic Enzyme Screening System (GESS), which is a whole-cell biosensor using genetic circuit, to identify novel enzymes from massive libraries [12,13]. GESS can screen various genes or microorganisms containing desired activity in a fluorescence-based assay using a flow cytometer (about 10^7 cells per hour) or product-induced quorum sensing. We applied the GESS to detect cellulase activity, but only examined artificial phenolic substrates [12].

In this study, we first created a cellobiose-detectible GESS (CBGESS) for sensing cellulolytic activity. The CBGESS recognizes cellobiose by CelR from *Thermobifida fusca* [14,15] and activates transcription of a reporter gene encoding fluorescent protein. Using the CBGESS, we could detect the activity of cellulase using cellobiose-dependent intracellular fluorescence with the help of a cocktail of endogenous cellulase and a commercial cellulase.

2. Materials and methods

2.1. Materials

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Restriction endonucleases, T4 DNA ligase and Phusion DNA polymerase were purchased from New England Biolabs (Ipswich, MA, USA). All oligonucleotides used in this study were synthesized by Bioneer Co. (Daejeon, Korea). DNA manipulation and transformation techniques were carried out according to standard protocols for molecular biology. Plasmid DNA isolation and DNA extraction were performed using a Qiagen Kit (Hilden, Germany). Celluclast 1.5L (Novozymes, Bagsvaerd, Denmark) was used as a cellulase cocktail to test crystalline cellulose hydrolysis. *Escherichia coli* BW25113 (KEIO collection), BW25113Δ*lacY::kan* (KEIO collection), MG1655, and DH5α were used as hosts for the cellobiose-biosensor.

2.2. CBGESS construction

To construct the CBGESS, pGESSv4 from a previous study was used as a backbone DNA template [12]. Briefly, the transcriptional regulator and reporter gene in pGESSv4 are located in opposite directions within promoters and terminators to avoid transcriptional noise. For cellobiose sensing, *celR*, a cellobiose-dependent regulator gene, was amplified by PCR from genomic DNA of *T. fusca* (ATCC BAA-629) [15]. The *celR* was subcloned under a strong and constitutive HCE promoter from pHCEIIB (Takara, Shiga, Japan). *sf-gfp* or *egfp* was used as a reporter gene and subcloned under a modified TRC promoter, in which the binding site of LacI was substituted with that of CelR (TGGGAGCGCTCCCA) using primer (Ptrc-R1) for cellobiose-dependent expression. The plasmid pCBGESS was constructed by ligation of DNA fragments into the plasmid pUC19 and transformation into *E. coli* DH5α, BW25113 and MG1655.

2.3. Cellulase construction and activity confirmation in vitro

For application of CBGESS in the cellulase assay, CelEdx16, which is a bifunctional endo/exo type cellulase, was used as a model enzyme [16]. *celEdx16* without a signal sequence was amplified by PCR and ligated into pPROEX-HTa (Invitrogen, Carlsbad, CA, USA). For low copy expression, the region of *celEdx16* and promoter from pPROEX-HTa was amplified and subcloned into pCC1-BAC (Invitrogen) by the Gibson assembly method. The subcloned CelEdx16 gene was transformed to DH5α and expressed in LB media with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 100 μg/mL ampicillin at 37 °C for 16 h. The cultivated cells were harvested by centrifugation at 1000 × *g* for 20 min, resuspended in 50 mM acetate buffer (pH 5.0), disrupted by sonication for 3 min and centrifuged at 15,000 × *g* for 20 min to remove cell debris. The supernatant was used directly to confirm the CelEdx16 activity assay which carried out for 1 h in 50 mM acetate buffer containing 200 μM *p*-nitrophenyl β-D-cellobioside ($pNPG_2$) or 0.1% carboxymethyl cellulose (CMC). Cellulase activity of CelEdx16 with CMC was measured by dynamic light scattering to identify reducing sugars [17]. To detect generated *p*NP or reducing sugars by cellulase activity, a multi-label reader (VictorX, PerkinElmer, Waltham, MA, USA) was used at absorbance 405 nm or 540 nm.

2.4. Fluorescent analysis of CBGESS

E. coli cells harboring pCBGESS were grown in Luria Bertani (LB) medium containing different concentrations of cellobiose and appropriate antibiotics at 37 °C for 10 h to detect of dose-dependent fluorescence signals. The fluorescence intensity of CBGESS was measured using a FACSCalibur (BD Biosciences, San Jose, CA, USA). A blue laser (Ex: 488 nm) and FL1 (Em: 530/30 nm) photo multiplier tube detector were used to analyze fluorescence signals at the single-cell level. Flow-cytometric data were analyzed using Flowjo software package (Flowjo, Ashland, OR, USA). To analyze fluorescence signal at the tube level, a multi-label plate reader was used at excitation and emission wavelengths of 485/10 and 535/20 nm. The Hill equation was used to analyze observed fluorescence data using Sigmaplot (Systat Software, Inc., Chicago, IL, USA). In the cell assay for various ligands, an extra 1 mM of ligands was added to the LB medium and fluorescence intensity was measured using the plate reader after 10 h incubation. In order to convert cellulase activity to fluorescence signal, *E. coli* cells harboring pCBGESS and the CelEdx16 gene were cultivated in LB containing 0.1 mM IPTG, 100 μg/mL ampicillin, and various ligands at 37 °C and monitored for 14 h.

For fluorescent analysis on LB agar plates containing different substrates, cells were incubated at 37 °C for 16 h and fluorescence was measured using a fluorescence microscope (Nikon AZ100-M, Tokyo, Japan) equipped with a GFP filter (excitation: 455–485 nm, emission: 500–545 nm).

To detect cellulase activity hydrolyzing crystalline cellulose on solid medium, cells harboring pCBGESS were spread on an LB plate containing 0.5% Avicel and 1 μL of Celluclast 1.5L (700 EGU/g, 60 FPU/mL, Novozymes) was dropped onto the plate. The plate was incubated at 37 °C for 16 h and used for measurement of fluorescence with a fluorescence microscope. For liquid experiments, cells harboring pCBGESS were reacted in LB broth containing 100 μg/mL ampicillin, 0.5% Avicel, and various concentrations of Celluclast 1.5L at 37 °C for 10 h and used for determination of fluorescence with the plate reader and fluorescence microscope.

3. Results and discussion

3.1. Design of cellulase biosensor

Cellulose was hydrolyzed to small carbohydrates including cellobiose by endo- or exo-type cellulase. To develop a cellulase biosensor, CelR from *T. fusca*, which is a cellobiose-dependent transcriptional regulatory protein, was used to construct a genetic circuit. CelR is a GalR-LacI family of transcriptional regulator that controls the expression of cellulase genes in *T. fusca* by cellobiose-induced affinity changes at specific DNA regions [15]. In the genome of *T. fusca*, upstream regions of six cellulase-encoding

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genes contain the same CelR binding sites, 14 base pairs of palindromic sequence (TGGGAGCGCTCCCA), and the expression of these genes is regulated by CelR. In this study, a modified TRC promoter, possessing substituted LacI binding site with that of CelR, was applied for cellobiose-based transcriptional regulation to achieve a definite phenotype in host expression systems. The schematic diagram of the CBGESS working principle with natural and artificial substrates is presented in Fig. 1. Both, endo- and exo-type cellulase can generate cellobiose as its products of enzymatic reactions, and artificial (*p*NPG2, MUG2, etc.) and natural substrates (wood, Avicel, CMC, etc.) would be used. The generated cellobiose from these substrates initiated the transcriptional activation of the *gfp* reporter by detaching CelR from the promoter region of the *gfp* reporter gene. Using intracellular fluorescent proteins in conjunction with flow cytometry or plate reader, high-throughput screening is possible to screen cellulase genes from various sources.

3.2. Characteristics of CBGESS

To detect cellobiose by the CBGESS whole-cell biosensor, cellobiose should be transported to the inside of cells and stably maintained to induce the CelR regulator. *E. coli* typically cannot utilize cellobiose as a sole carbon source because operons involving the cellobiose-metabolic pathway (*chb* and *asc*) are cryptic [18]. These cryptic operons are regulated to be phenotypically silent under natural conditions by transcriptional regulation systems, which are not induced by cellobiose. Previously, LacY (lactose permease) was reported to be a low affinity cellobiose transporter in the *E. coli* KO11 strain [19]. Thus, we tested the CBGESS in a *lacY*-deleted *E. coli* BW25113 from the KEIO collection, and the biosensor showed quantitative responses to cellobiose compared to that of the wild-type strain (Fig. S1). This result suggests that cellobiose uptake in *E. coli* is associated with not only LacY, but also some other factors at low concentrations of cellobiose that are available to activate CBGESS.

To determine the best *E. coli* strain for CBGESS, we tested MG1655 and DH5α strains. DH5α showed a quantitative and uniform fluorescence signal under all tested concentrations of cellobiose in flow cytometric analysis whereas MG1655 showed heterologous populations; non-fluorescent (major population) and fluorescent cells (minor population). (Fig. S2). There was no significant difference in genotypes between MG1655 and DH5α when it comes to glucan transporter or glucanase. Although only MG1655 contains *lacZ* gene encoding β-galactosidase, the enzyme cannot degrade intracellular cellobiose. However, alterations in membrane permeability due to mutations in membrane-associated genes in DH5α may contribute to sensitive detection of cellobiose of CBGESS [20].

To determine the dynamic range of cellobiose detection by CBGESS, fluorescence signals from DH5α harboring pCBGESS were measured at different concentrations of cellobiose ranging from 0 to 100 μM in LB media (Fig. 2a and b). In flow cytometric analysis, the CBGESS showed strong and proportional fluorescence signals against various concentrations of cellobiose (Fig. 2a). The highest mean fluorescence intensity was approximately 50-fold higher than that of cultivated cells without cellobiose. The high signal-to-noise ratios and cellobiose-dependent fluorescence intensity indicated that the CBGESS is an effective biosensor for detecting cellulase activity at the single cell level.

The biosensor showed dose-response fluorescence to cellobiose, and the fluorescent linearity was obtained in the range of 5–30 μM (Fig. 2b). The dose-response curve represents non-linear regression fit of the fluorescent data to the four-parameter Hill equation, as described in Eq. (1).

$$y = \left\{ (y_{max} - y_{min})[cellobiose]^n \,/\, K_{1/2}^n + [cellobiose]^n \right\} + y_{min} \quad (1)$$

where y is the fluorescence intensity at a given concentration of cellobiose, y_{max} is the fluorescence intensity at the saturating concentration of cellobiose, and y_{min} is the fluorescence intensity at a cellobiose concentration of zero. $K_{1/2}$ is the concentration of cellobiose at which a half-maximal effect is observed and n is the Hill coefficient (n_{app}) describing assay sensitivity [21]. Values of 18.70 ± 0.55 μM for $K_{1/2}$ and 1.76 ± 0.08 for n_{app} were obtained from the curve fit. These values are comparable to other transcriptional regulators including LacI at the same TRC promoter [21]. Therefore, CBGESS can be applied not only for sensing cellobiose as a biosensor, but also for expression of recombinant proteins as an induction system like LacI-based expression system.

The ligand specificity of CBGESS was examined using various glucose polymers (Fig. 2c) or disaccharides (Fig. 2d). Among bi-to penta-glucose polymers with β-1,4-glycosidic bonds, only cellobiose induced strong intracellular fluorescent response in cells harboring pCBGESS (Fig. 2c). In the test of disaccharide-inductions, the CBGESS only showed strong fluorescent signal in the presence of cellobiose (Fig. 2d). The ligand-specificity of CBGESS is affected by the characteristics of CelR and dependency on the transport systems of the host. This cellobiose specific recognition may be a beneficial effect for detecting exo-type glucanase activity, which reacts at the edge of cellulose (reducing or non-reducing end) and generates cellobiose as its enzymatic product.

3.3. Application of CBGESS to detect cellulolytic activity

To probe the applicability of CBGESS to screen cellulases, exo/

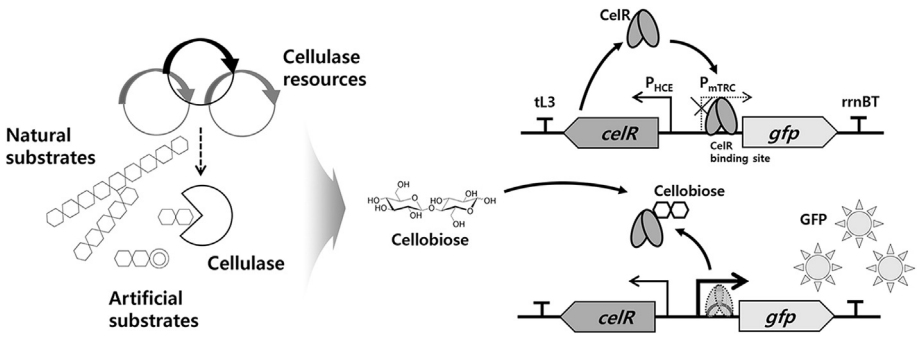

Fig. 1. Schematic diagram of CBGESS working principle. Under natural conditions, CelR along with its constitutive promoter repressed transcriptional initiation of the *gfp* by binding to the CelR recognition site in the 5′-upstream region of the *gfp*. When cellobiose is generated from natural or artificial substrates by cellulase from various resources, the fluorescent protein is produced by releasing the CelR-cellobiose complex.

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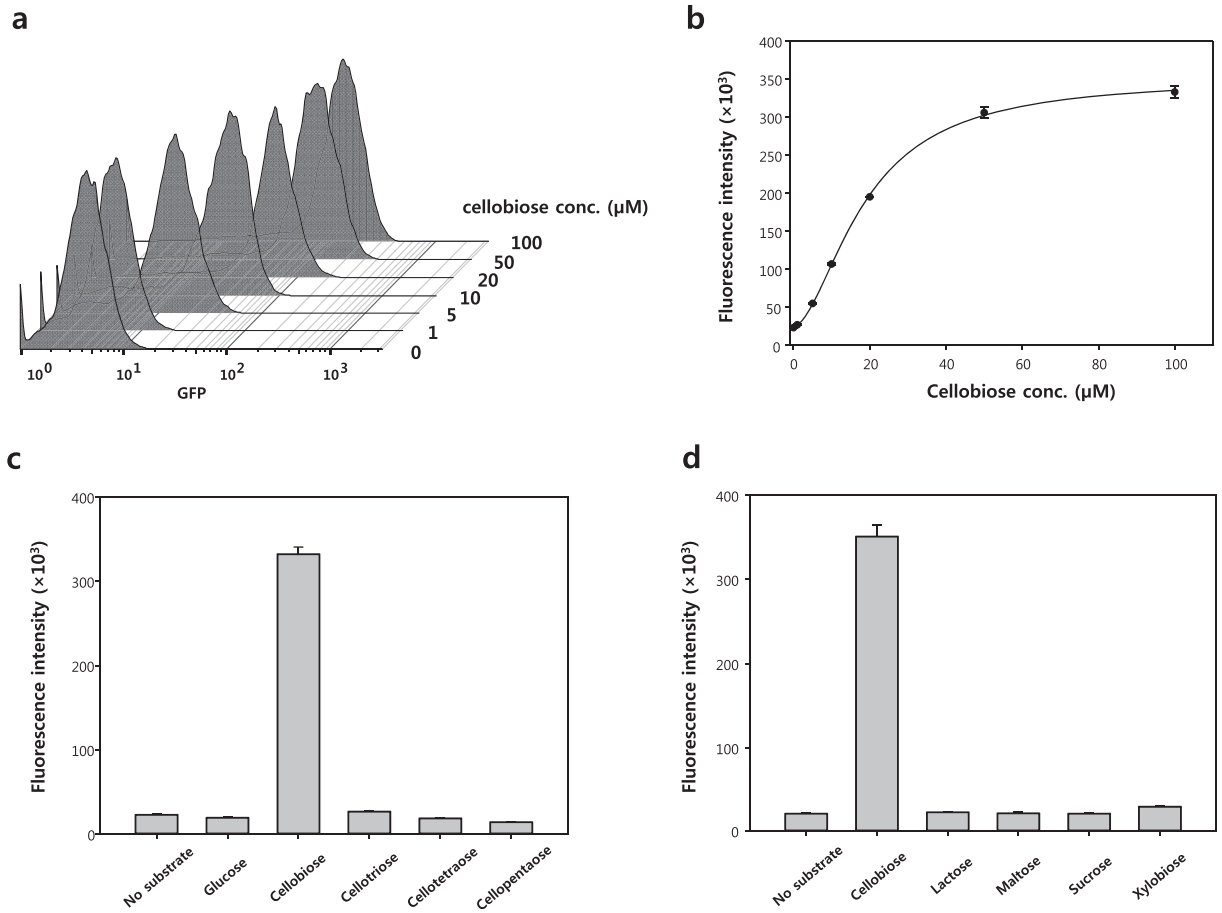

Fig. 2. Fluorescence response of CBGESS to cellobiose. **a**. Flow cytometric analysis of cells harboring pCBGESS at different concentrations of cellobiose. **b**. Dose-response curve of CBGESS with cellobiose. Normalized fluorescence intensity (Fluorescence/OD_{600nm}) was analyzed with a multi-label plate reader. **c**. Fluorescence response of CBGESS at various glucose polymers. Each compound was used at 1 mM concentration. Bi-to penta-glucose units formed β-1,4 linkage by glycosidic bond. **d**. Ligand specificity of CBGESS in the presence of different disaccharides.

endo dual functional cellulase (CelEdx16) [16] was used as a model enzyme. CelEdx16 hydrolyzed not only CMC as an endo-type model substrate, but also *p*NPG_2 as an exo-type model substrate (Fig. S3). CelEdx16 randomly hydrolyzes in the middle of CMC and produces cello-oligosaccharides including cellobiose. *p*NPG_2 is degraded by CelEdx16 at its non-reducing ends and generated *p*NP and cellobioside. CBGESS cells expressing CelEdx16 gene were cultivated with CMC or *p*NPG_2 in LB and intracellular fluorescence intensity was measured using a fluorescent reader, fluorescent microscope, and flow cytometer (Fig. 3). In time-course fluorescence profiles by fluorescent reader, CBGESS responded to cellobiose, as indicated by GFP expression within 3 h of induction, while cells in the absence of any substrate showed no fluorescent signal (Fig. 3a). Different response times (cellobiose, 3 h < CMC, 7 h < *p*NPG_2, 8 h) and fluorescence intensities (cellobiose > CMC > *p*NPG_2) were observed for CBGESS cells cultivated with cellobiose, CMC or *p*NPG_2. Because this may be due to the difference in CelEdx16 activity against different substrates, these various response times and intensities are a measurable parameter for characterizing cellulase activity.

In the flow cytometric assay, CBGESS cells expressing CelEdx16 enzyme showed significantly high fluorescence signals in the presence of all three substrates, whereas the same cells lacking the CelEdx16 cellulase as a negative control did not produce intracellular fluorescence (Fig. 3b). These results suggest that CBGESS with flow cytometry can be applied to high-throughput screening systems at the single cell level.

After 16 h cultivation on LB solid agar plates containing each substrate, colonies harboring both CBGESS and CelEdx16 generated strong fluorescence signals in fluorescent microscope analysis (Fig. 3c), as was observed in flow cytometric analysis. Particularly, plate-based assays have advantages for cellulase screening in terms of utilizing insoluble substrates and minimizing product diffusion. CBGESS was successfully applied to detect cellulase activity *via* fluorescence signals both in liquid and solid cultures, which showed various response times and fluorescence intensities in accordance with different substrates used. Moreover, high-throughput screening for new cellulases is possible with this CBGESS using flow cytometry or with a solid plate containing insoluble substrates.

3.4. Application of CBGESS for detecting crystalline cellulose hydrolysis

Crystalline cellulose has representative biomass recalcitrance that is resistant to enzymatic hydrolysis. Therefore, finding novel cellulases that can efficiently hydrolyze crystalline cellulose may overcome bottlenecks of cellulose utilization in the biomass-based industry. To demonstrate the usefulness of CBGESS for detecting the hydrolysis activity of crystalline cellulose by cellulase, CBGESS cells were cultivated in LB plate containing a crystalline substrate, Avicel

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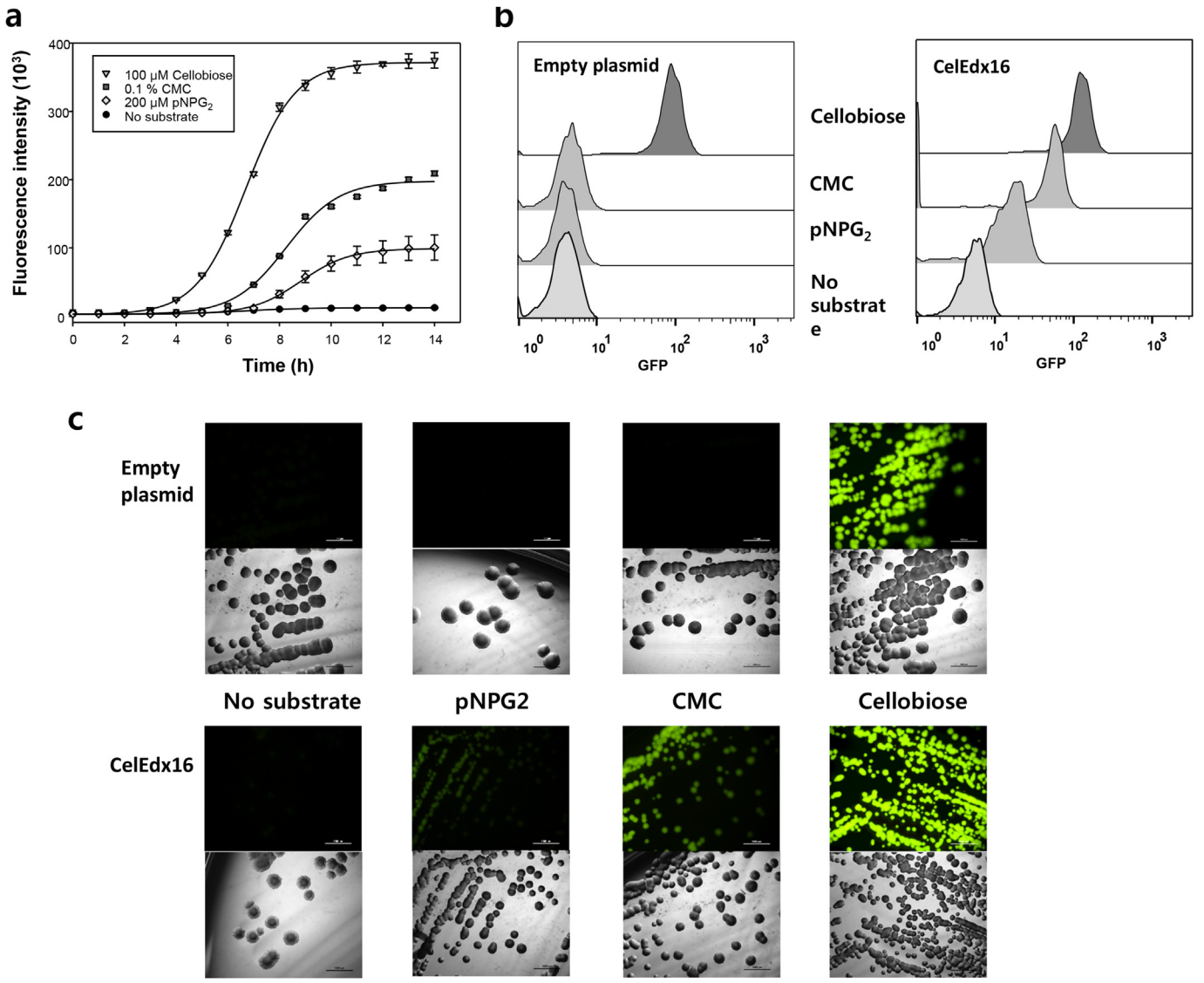

Fig. 3. Quantitative fluorescence analysis of cellulase activity using CBGESS. **a**. Time-course fluorescence profile of cells harboring cellulase and pCBGESS with different substrates. **b**. Flow-cytometric analysis of cellulase activity *via* CBGESS. Empty plasmid indicates cells harboring pCBGESS and empty plasmid without cellulase gene, while CelEdx16 indicates cells containing pCBGESS and cellulase gene. **c**. Fluorescence microscopic analysis of cellulase activity detected by CBGESS on LB agar-plates containing different substrates.

(0.5%), and Celluclast 1.5L was dropped onto CBGESS colonies (Fig. 4). Celluclast 1.5L is a commercial cellulase cocktail that can hydrolyze crystalline cellulose and produce cellobiose without β-glucosidase. In solid-phase experiments, CBGESS cells nearby with Celluclast 1.5L showed strong green fluorescence signals that are representative of crystalline cellulose degradation, while CBGESS cells nearby with dropped phosphate-buffered saline showed no signals (Fig. 4a). The boundary of fluorescence signal was defined by the amount of cellulase and solid plate as a physical diffusion barrier.

To determine the dose-dependent fluorescence response of CBGESS by the Celluclast 1.5L, CBGESS cells were incubated in LB broth containing 0.5% Avicel and different concentration of Celluclast 1.5L (Fig. 4b). In fluorescence analysis using a plate reader and microscopy, fluorescence intensity increased with the increase in cellulase concentration, i.e., CBGESS responded quantitatively to the cellobiose as a product from crystalline cellulose hydrolysis with different cellulase concentrations. In many cellulolytic organisms, exoglucanase hydrolyzes crystalline cellulose and cellobiose is generated as the product [4]. Therefore, CBGESS can be used as an exo-type cellulase-specific biosensor using crystalline cellulose. The restrictive condition of this biosensor-based screening is the accessibility of the substrate to cellulase. In solid-phase systems, we previously reported antibiotic-free high-throughput screening of WT microorganisms using a biosensor for detecting enzyme/cells activities with surrounding sensor cells through "cell-to-cell communications" [13]. This CBGESS also can be applied with cell-to-cell communication to screen WT microorganisms or fungi for extracellular enzymes. CBGESS is a new screening platform for detecting cellulase, particularly crystalline cellulose-hydrolyzing exoglucanase.

4. Conclusions

In this study, we developed a cellobiose-detecting whole-cell biosensor consisting of a cellobiose-dependent regulator and fluorescent reporter protein that allow for precise determination of cellulase activity inside living cells. CBGESS is a powerful tool for

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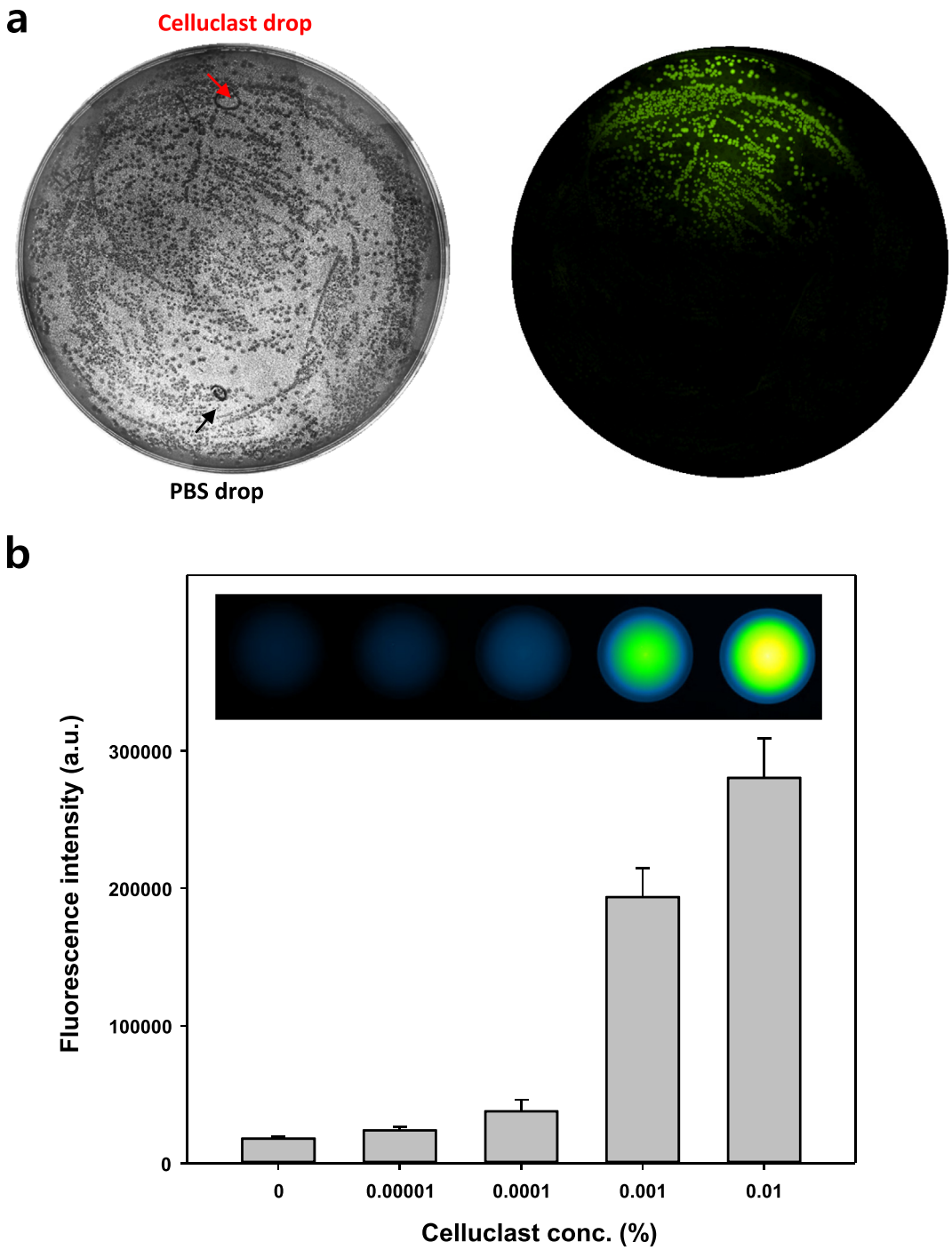

Fig. 4. Detection of crystalline cellulose hydrolysis by CBGESS. **a**. Fluorescence microscopic image of CBGESS colonies. Cells were cultivated on an LB plate containing 0.5% Avicel. Red arrow indicates position of dropping Celluclast 1.5L and black arrow is PBS as a control. **b**. Fluorescence analysis of hydrolyzing 0.5% Avicel in the presence of various concentrations of Celluclast 1.5L using CBGESS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sensing cellulase with high sensitivity inter- and intra-cellularly. Furthermore, the CBGESS can be used to detect the activity of cellulase hydrolyzing crystalline cellulose by measuring its fluorescence intensity. This whole-cell biosensor is a useful screening system for novel cellulase or cellulolytic organisms from both metagenomic libraries and different natural environments.

Disclosure and conflicts of interest

The authors declare that they have no disclosure and conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2017.11.157.

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