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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

A *Pichia pastoris* single-cell biosensor for detection of enzymatically produced methanol

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Abstract

We conducted single-cell analyses of the methylotrophic yeast *Pichia pastoris* to develop a biosensor for the detection of methanol produced by heterologous enzymes. In this biosensor, methanol and its subsequent metabolism induce expression of a gene encoding a fluorescent protein that was placed under the control of a methanol-inducible promoter. Using quantitative analyses of fluorescence microscopy images, a methanol-inducible promoter and a host strain were selected, and preculture and assay conditions were optimized to improve the methanol detection limit. Fluorescence-activated cell sorting (FACS) analysis of the distribution and geometric mean of cellular fluorescence intensity against various concentrations of methanol revealed a detection limit of 2.5 μM. Finally, this biosensor was applied to evaluate the activity of a heterologously expressed pectin methylesterase (PME). The cellular fluorescence intensity was proportional to the copy number of the PME expression cassette, the protein level, and the enzyme activity. This biosensor can be used for high-throughput screening of single cells harboring high methanol-producing activity, and thereby, the development of a bioconversion process using methanol-producing enzymes.

Keywords Single-cell biosensor · Methanol-producing enzyme · *Pichia pastoris* · Pectin methylesterase · Methanol-inducible gene expression

Introduction

In bioconversion processes using recombinant enzymes in microbial cells, the productivity of the target compound depends largely on the activity and stability of the relevant enzyme being produced. To be successful, it is critical to develop a high-throughput system for screening microbial cells with high enzyme activity and/or stability. Recently, approaches using single-cell analyses have enabled us to conduct such high-throughput screening at a single-cell level. If a biosensor that produces fluorescence in response to the reaction product of the recombinant enzyme is created, cells having higher enzyme activity can be selected at a single-cell level in a high-throughput manner, e.g., via fluorescence-activated cell sorting (FACS) technology (DeLoache et al. 2015; Ho et al. 2017; Jha et al. 2014; Michener et al. 2012; Mustafi et al. 2012).

Methanol is industrially used as a solvent and is a feedstock for various chemicals and value-added products, e.g., plastic materials and pharmaceuticals (Schrader et al. 2009). While methanol is currently produced from syngas (CO/H_2), its direct production from biomass or natural gas has been a long-time focus in industry (Olah et al. 2009). Methanol-producing enzymes, such as pectin methylesterase (PME) and methane monooxygenase, are expected to be used for methanol production from pectin (in plant biomass) and from methane (a main component of natural gas), respectively.

To exploit the potential of methanol-producing enzymes for bioconversion processes, a sensitive method to evaluate their activities at a single-cell level should be established. So far, several biosensors for methanol detection have been reported with microbial cells or enzymes immobilized on an oxygen electrode (Guilbault et al. 1983; Wen et al. 2014).

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However, these biosensors cannot be used for the detection of methanol endogeneously produced by recombinant enzymes. We and others previously described biosensors for methanol detection based on the expression of a fluorescent protein gene under the control of a methanol-inducible promoter (Abanda-Nkpwatt et al. 2006; Kawaguchi et al. 2011). In these fluorescent biosensors, the fluorescence intensity of single cells was analyzed with fluorescence microscopy. However, the detection limit needed to be improved to detect enzymatically produced levels of methanol for application to high-throughput analysis such as FACS, and the feasibility for evaluation of methanol-producing enzyme activity had not been investigated.

In this study, we first developed a single-cell biosensor for methanol detection with the methylotrophic yeast *Pichia pastoris* (currently reclassified as *Komagataella phaffii*), which has been widely used as a host for heterologous protein production (Ahmad et al. 2014; Gellissen 2000). Methylotrophic yeasts, capable of utilizing methanol as the sole carbon and energy source, have methanol-sensing machinery to respond to widely fluctuating methanol concentrations in nature as well as in laboratory culture (Kawaguchi et al. 2011; Ohsawa et al. 2017). Figure 1 shows the design of the new methanol biosensor. Expression of a gene encoding a fluorescent protein under the control of a methanol-inducible promoter is induced by methanol in a dose-dependent manner and completely repressed by the presence of glucose. Selection of the promoter, optimization of sensing conditions, and analysis of the distribution of cellular fluorescence intensity by FACS improved the limit of detection for methanol to the micromolar range.

We applied the methanol sensor cells as the host for production of the heterologous methanol-producing enzyme pectin methyl esterase (PME) from *Aspergillus niger* (Kawaguchi et al. 2014), and thereby, to the detection of its activity. The enzyme assay conditions for the methanol sensor would be quite different from the conditions for detection of exogenous methanol present at the start of incubation, in that the concentration of methanol may change during cellular metabolism. We confirmed that the methanol sensor could detect enzyme activity as a result of methanol formation by PME, even though the concentration of methanol may not have reached the detection limit at any point. Our results show the feasibility of the newly developed methanol sensor for evaluating the activity of methanol-producing enzymes.

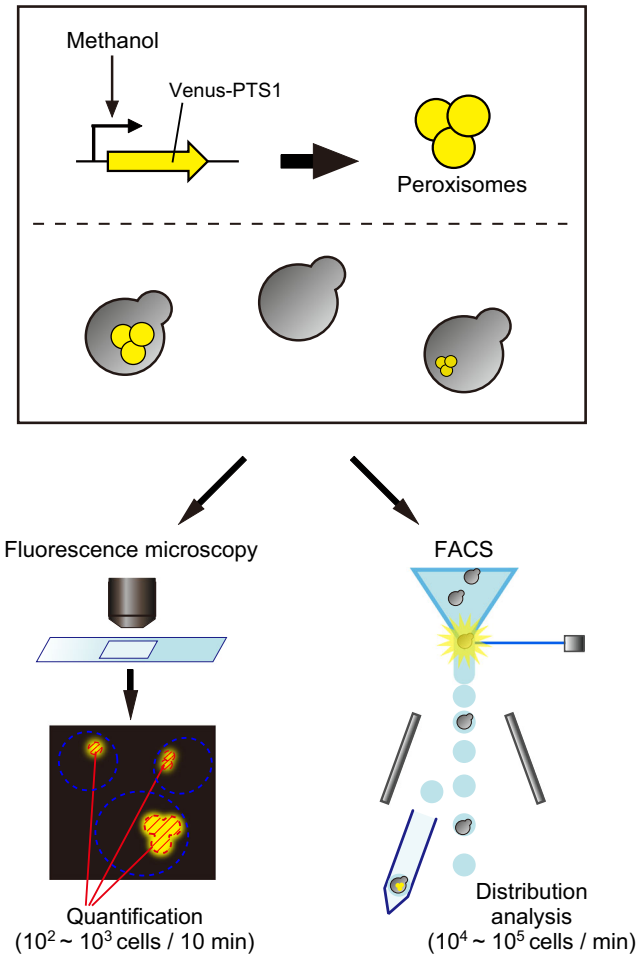


Fig. 1 Schematic illustration of the *P. pastoris* single-cell methanol sensor. The fluorescent protein Venus tagged with peroxisome targeting signal 1 (Venus-PTS1) is expressed under the control of a methanol-inducible promoter. These cells show methanol-dependent fluorescence in peroxisomes. Fluorescence microscopy enables visual assessment of the cellular response, and the cellular fluorescence intensity can be determined by image analysis (10^2~10^3 cells per 10 min). FACS analysis enables high-throughput analysis and sorting of single cells (10^4~10^5 cells/min)

Materials and methods

Strains and media

The yeast strains used in this study are listed in Table 1. *P. pastoris* cells were grown at 28 °C on glucose medium (1% yeast extract, 2% peptone, 2% glucose). The assay media for the detection of methanol (methanol medium) contained 0.67% yeast nitrogen base without amino acids (YNB w/o a.a.) and 0–2.5 × 10^5 μM methanol. The assay medium for detection of PME activity (PME assay medium) contained 0.67% YNB w/o a.a. and 0.05% pectin (esterified, from citrus fruit, Sigma, St. Louis, MO). All of the components in these media other than carbon sources were purchased from Becton Dickinson (Franklin Lakes, NJ). Cell growth was monitored by the optical density at 610 nm (OD_{610}).

Plasmid construction and transformation

The oligonucleotide primers used in this study are listed in Table S1. The plasmids used in this study are listed in Table 2. pAOX1V was obtained by inserting the 0.7-kb

Table 1 Yeast strains used in this study

Strain	Genotype	Description	Source or reference
PPY12	*his4 arg4*	Wild type	ATCC 204163 (Gould et al. 1992)
PMT1101	PPY12 *his4*::pAOX1V (P_{AOX1}-Venus-PTS1 *HIS4*)	Auxotrophic methanol sensor candidate strain	This study
PMT1301	PPY12 *his4*::pDAS2V (P_{DAS2}-Venus-PTS1 *HIS4*)	Auxotrophic methanol sensor strain	This study
PMT1102	PMT1101 *arg4*::pNT204 (*ARG4*)	Non-auxotrophic methanol sensor candidate strain	This study
PMT1302	PMT1301 *arg4*::pNT204 (*ARG4*)	Non-auxotrophic methanol sensor strain	This study
PMT1303	PMT1302 P_{GAP}::pGAPZPME (P_{GAP}-PME-His_6 *Sh ble*)	Methanol sensor strain with a single copy of PME-His_6	This study
PMT1304	PMT1303 P_{GAP}::pGAPBPME (P_{GAP}-PME-His_6 *Blasticidin*)	Methanol sensor strain with two copies of PME-His_6	This study
PMT1305	PMT1303 P_{ACT1}::pACTBmC (P_{ACT1}-mCherry *Blasticidin*)	Methanol sensor strain with a single copy of PME-His_6 labeled with mCherry	This study
KM71	*his4 arg4 aox1*Δ::*ScARG4*	*aox1*Δ	Invitrogen
PMT1401	KM71 *his4*::pDAS2V (P_{DAS2}-Venus-PTS1 *HIS4*)	Methanol sensor strain with *aox1*Δ background	This study

Venus gene (Nagai et al. 2002) fused to the peroxisome targeting signal 1 (PTS1) sequence (Ser-Lys-Leu) into the *Kpn*I/*Bam*HI sites of pIB4 (Sears et al. 1998). pDAS2V was constructed by removing the promoter region of pAOX1V between the *Aat*II/*Sac*I sites, where the 1.0-kb upstream region of the *DAS2* gene was inserted. pAOX1V and pDAS2V were linearized at the *Stu*I site for transformation. To construct pGAPZPME, a 996-bp DNA fragment corresponding to the deduced amino acid sequence of the *A. niger pmeA* gene (Kawaguchi et al. 2014), which includes an N-terminal secretory signal peptide, was inserted into the *Eco*RI/*Not*I sites of pGAPZB in frame with a C-terminal His_6-tag encoding sequence in the plasmid. The PME-His_6 expression cassette of pGAPZPME was excised with *Nsi*I and *Bam*HI and inserted into the *Pst*I/*Bam*HI sites of pTEF1/Bsd to obtain pGAPBPME. pGAPZPME and pGAPBPME were linearized at the *Avr*II site for transformation. *P. pastoris* cells were transformed by electroporation as described previously (Wu and Letchworth 2004).

Fluorescence microscopy and image analysis

Non-auxotrophic strains were derived by introduction of the corresponding auxotrophic gene, and used for image and FACS analysis. Cells were observed with a motorized inverted microscope (IX81; Olympus, Tokyo, Japan) equipped with an Uplan-Apochromat 100×/1.35 NA oil iris objective lens. Venus signal was acquired using 500AF20, 455DRLP, and 535AF30 filter sets (Omega Optics, Austin, TX). Image data were captured with a charge-coupled device camera DP30BW (Olympus) at a fixed exposure time of 100 ms. For each sample, one field with at least 100 cells was captured. Data were acquired and analyzed by using MetaMorph software version 7.0 (Molecular Devices, San Jose, CA) and saved as 16-bit TIFF files. Quantification of fluorescence intensity was conducted with ImageJ software version 1.51n (https://imagej.nih.gov/ij/) as follows. The threshold was set from 145 to 4095 to measure the fluorescence intensity of pixels covering peroxisomes. Fluorescence intensity of the pixels

Table 2 Plasmids used in this study

Plasmid	Description	Source or reference
pIB4	*HIS4*; P_{AOX1}-based expression vector	(Sears et al. 1998)
pAOX1V	Venus-PTS1 inserted into *Kpn*I/*Bam*HI sites of pIB4	This study
pDAS2V	1.0-kb upstream region of the *DAS2* gene inserted into *Aat*II/*Sac*I sites of pAOX1V	This study
pNT204	*ARG4*	(Tamura et al. 2010)
pGAPZB	$Zeocin^R$; P_{GAP}-based expression vector	Invitrogen
pGAPZPME	*A. niger pmeA* inserted into *Eco*RI/*Not*I sites of pGAPZB in frame with a C-terminal His_6-tag sequence	This study
pTEF1/Bsd	$Ampicillin^R$, $Blasticidin^R$	Invitrogen
pGAPBPME	P_{GAP}-PME-His_6 expression cassette of pGAPZPME inserted into *Pst*I/*Bam*HI sites of pTEF1/Bsd	This study
pACTBmC	P_{ACT1}-mCherry expression cassette inserted into *Bam*HI/*Eco*RI sites of pTEF1/Bsd	This study

within the set threshold was averaged to represent the cellular fluorescence intensity of the image (expressed as arbitrary units, a.u.). Three independent experiments starting from distinct single colonies were conducted.

Flow cytometry

Cells were resuspended in ice-cold phosphate buffered saline (PBS) buffer and kept on ice. Flow cytometry was performed using FACSAria IIIu (Becton Dickinson). Fluorescent channel and light scatter were set at log gain. The forward scatter (FSC) was set at a photomultiplier tube (PMT) voltage of 250 with a threshold of 2000. The side scatter (SSC) PMT voltage was set at 250. Venus fluorescence was excited with a 488 nm laser, and the emission at 530/30 nm was detected at a PMT voltage of 700 or 900. At least 30,000 cells were analyzed per sample. Cells were gated for forward and side scatter to select single cells as shown in Fig. 3a–c. Since Venus fluorescence intensity (Venus-A) was proportional to forward scatter (FSC-A), which reflects cell size (Fig. 3d), Venus-A normalized to FSC-A (Venus-A/FSC-A) with 25% scaling was used to represent the fluorescence intensity of each cell. FACSDiva 8 software (Becton Dickinson) was used for data acquisition and creation of scatter plots. Bioconductor (www.bioconductor.org) in the open-source statistical platform R (www.r-project.org) was used for processing of FCS data, followed by preparation of histograms.

Immunoblot analysis

Cells were grown in glucose medium, and the amount of PME-His_6 secreted into the supernatant was analyzed as follows. The supernatant was mixed with 3× sample buffer (50 mM Tris-HCl pH 6.8, 30% glycerol, 3% SDS, 3% 2-mercaptoethanol, a dash of bromophenol blue) and boiled for 5 min. Resulting samples were electrophoresed on a 12% SDS-PAGE gel. Proteins were transferred onto a PVDF membrane by semidry blotting (Bio-Rad, Richmond, CA). The blot was blocked and incubated with anti-His-tag mAb-HRP-DirecT antibody (Medical & Biological Laboratories, Nagoya, Japan) at a 1:10,000 dilution for 1 h. The bound HRP-conjugated antibody was detected using Western Lightning (Perkin-Elmer Life Science, Waltham, MA), and the signal was analyzed with a Light Capture System (ATTO). PME-His_6 signal intensity was quantified using CS Analyzer software (ATTO).

PME enzyme activity assay

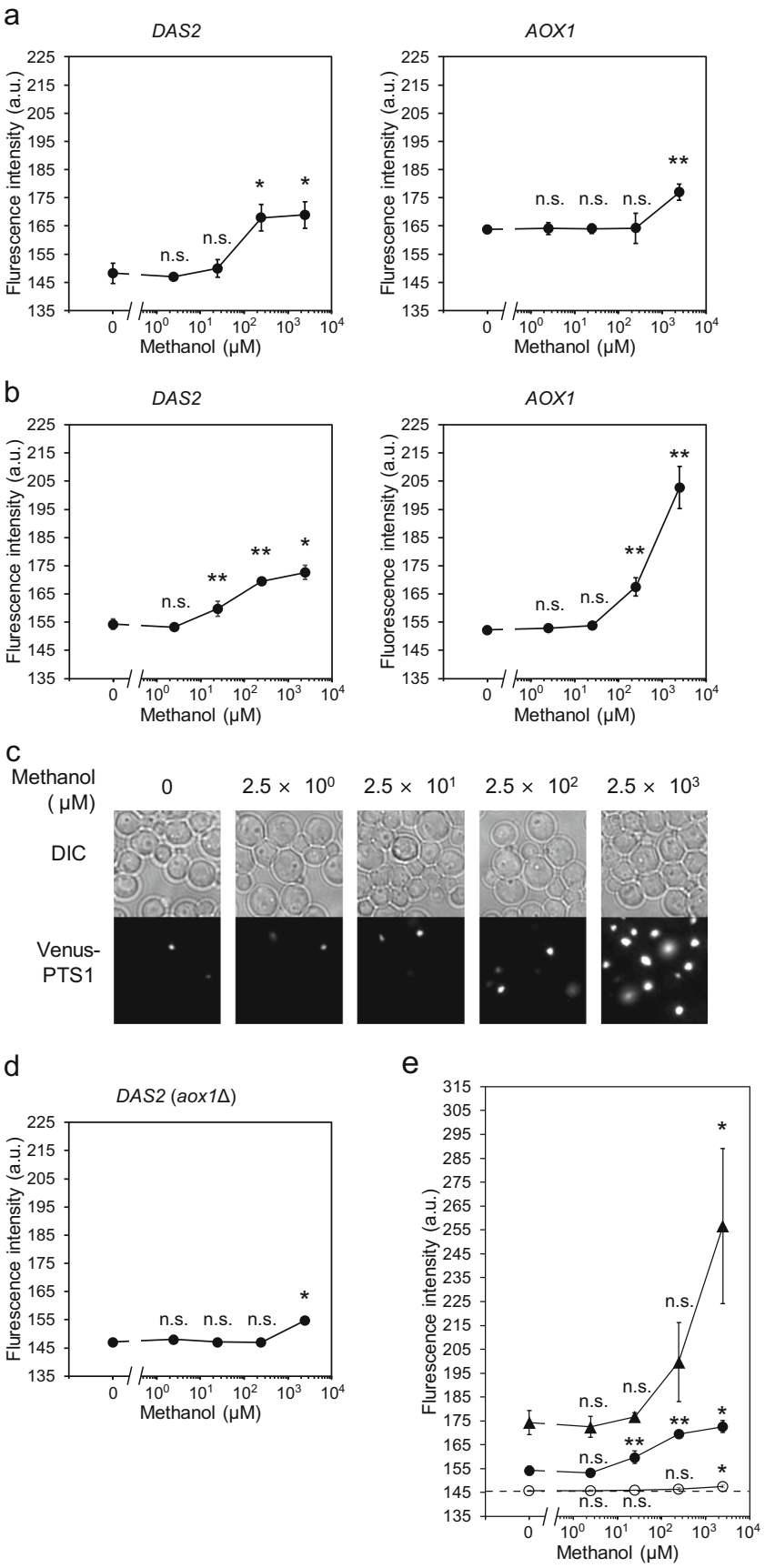
PME enzyme activity in the culture broth was determined as described previously (Anthon and Barrett 2004) with slight modifications. The reaction mixture contained 10 mM citrate buffer (pH 4.0), 0.5% (*w*/*v*) pectin and an appropriate amount of culture broth. This mixture was kept at 28 °C, and 100 μL was sampled at several time points and placed on ice to stop the reaction. Samples were mixed with an equal volume of 20 U/mL alcohol oxidase (from *P. pastoris*; Sigma) in 100 mM HEPES-KOH pH 8.0 and incubated at 37 °C for 10 min to oxidize the produced methanol to formaldehyde. The resulting mixture was mixed with an equivalent volume of 5 mg/mL Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) dissolved in 0.5 N NaOH. The condensation of formaldehyde and Purpald results in the formation of a cyclic aminal, which is subsequently oxidized to form a purple tetrazine dye. After 30 min of incubation at room temperature, absorbance at 550 nm was determined. A standard curve was generated from known concentrations of methanol. One unit of activity was defined as the amount of enzyme that produced 1 μmol of methanol per min.

Results

Construction of the *Pichia* methanol sensor strain and optimization of assay conditions

P. pastoris strains expressing the yellow fluorescent protein Venus (Nagai et al. 2002) tagged with peroxisome targeting signal 1 (PTS1) under the control of strong and methanol-inducible promoters, P_{DAS2} (dihydroxyacetone synthase gene) and P_{AOX1} (alcohol oxidase gene) (Liang et al. 2012; Stewart et al. 2001; Vogl et al. 2016) were constructed. Since peroxisome proliferation is coupled with methanol-induced expression of peroxisomal enzymes, Venus was targeted to peroxisomes,

Fig. 2 Development of a *P. pastoris* single-cell methanol sensor using fluorescence microscopy. **a** Comparison of the dose-response of two methanol-inducible promoters, P_{DAS2} and P_{AOX1}, using cells precultured on glucose medium to mid-exponential phase (Fig. S1) and transferred to medium containing various concentrations of methanol. Cells were observed after 4 h of incubation. **b** Similar analyses using cells precultured on glucose medium to late-exponential phase (Fig. S1). **c** Fluorescent images of methanol sensor cells using P_{DAS2} transferred from late-exponential phase preculture on glucose medium to the indicated methanol medium, together with differential interference contrast (DIC) microscopic images. **d** Influence of *AOX1* gene disruption on the dose-response of methanol sensor cells. Cells were precultured and analyzed as in Fig. 2b. **e** Time-course analysis of the dose-response. Cells were precultured and transferred into methanol medium as in **b**. Cells sampled at 2 h (open circles), and 6 h (closed triangles) during incubation in methanol medium were analyzed and compared with 4 h (closed circles) in **b**. Dashed line indicates the fluorescence intensity level at 0 h without methanol (145.62 ± 0.05). Fluorescence intensity data are expressed in arbitrary units (a.u.) and represent means ± s.d. of three independent experiments. Each experiment consisted of a glucose culture starting from a distinct single colony followed by transfer to methanol medium, resulting in one sample for each concentration. For each sample, the cellular fluorescence intensity was calculated from the image data of one field containing at least 100 cells. Statistical significance against the data at 0 μM methanol was calculated by a two-tailed paired *t* test (n.s., $p > 0.05$; *$p < 0.05$; **$p < 0.01$)


a
DAS2
AOX1
Flurescence intensity (a.u.)
Fluorescence intensity (a.u.)
Methanol (μM)
n.s.
*
**
b
DAS2
AOX1
c
Methanol (μM)
0
2.5 × 10^0
2.5 × 10^1
2.5 × 10^2
2.5 × 10^3
DIC
Venus-PTS1
d
DAS2 (aox1Δ)
e

which could give an intense punctate fluorescence signal distinguishable from the cytosolic background.

To repress the expression of the reporter Venus-PTS1 protein, cells were first grown on glucose medium and harvested at mid- or late-exponential phase (Fig. S1), and the effect of the growth phase on expression was examined. Harvested cells were transferred into medium containing various concentrations of methanol (0–2.5×10^3 μM) (methanol medium). After 4 h of incubation, at least 100 cells from each sample were observed in one field under a fluorescence microscope and the cellular fluorescence intensity was quantified using ImageJ software ("Materials and methods" section). The detection limit of the strain is defined as the lowest concentration of methanol showing cellular fluorescence intensity statistically higher than that without methanol.

When the mid-exponential phase cells grown in glucose medium were used for analysis, cellular fluorescence intensity with P_{DAS2} at 250 μM methanol, but not 2.5 or 25 μM, was significantly higher than that without methanol (Fig. 2a (left), $p < 0.05$). When P_{AOX1} was used, the cellular fluorescence intensity at 2.5×10^3 μM methanol, but not 2.5–2.5×10^2 μM, was significantly higher than that without methanol (Fig. 2a (right), $p < 0.01$). Therefore, the detection limit of the strain using P_{DAS2} (2.5×10^2 μM) was lower than that of the strain using P_{AOX1} (2.5×10^3 μM).

When late-exponential phase cells were used for analysis, cellular fluorescence intensity with P_{DAS2} at 25 μM methanol was significantly higher than that without methanol (Fig. 2b (left), $p < 0.01$) and increased as the concentration of methanol increased up to 2.5×10^3 μM (Fig. 2b (left) and c). Thus, the detection limit and dose dependency of methanol was improved by using late-exponential phase cells. A similar experiment using the strain with P_{AOX1} resulted in a detection limit of 2.5×10^2 μM, confirming that P_{DAS2} shows a lower detection limit under these conditions as in the case using mid-exponential phase cells (Fig. 2b, right). Accordingly, the strain expressing Venus-PTS1 under the control of P_{DAS2} was designated as the methanol sensor strain, and late-exponential phase cells were used in subsequent experiments.

We also tested a methanol sensor strain with an *aox1*Δ background with the expectation that disruption of the alcohol oxidase gene would diminish methanol consumption and result in an improved detection limit. However, the detection limit of this strain was 2.5×10^3 μM (Fig. 2d, $p < 0.05$). This observation is consistent with results of our previous study with another methylotrophic yeast, *Candida boidinii*, in which deletion of the alcohol oxidase gene (*AOD1*) resulted in a decrease in *AOD1* promoter activity (Sakai et al. 1999). Therefore, not only methanol itself but also its metabolism, yielding metabolites and energy, are considered to be necessary for full induction of Venus-PTS1 under control of the *DAS2* promoter. Accordingly, the wild-type methanol sensor strain, rather than the *aox1*Δ strain, was used in the following analyses.

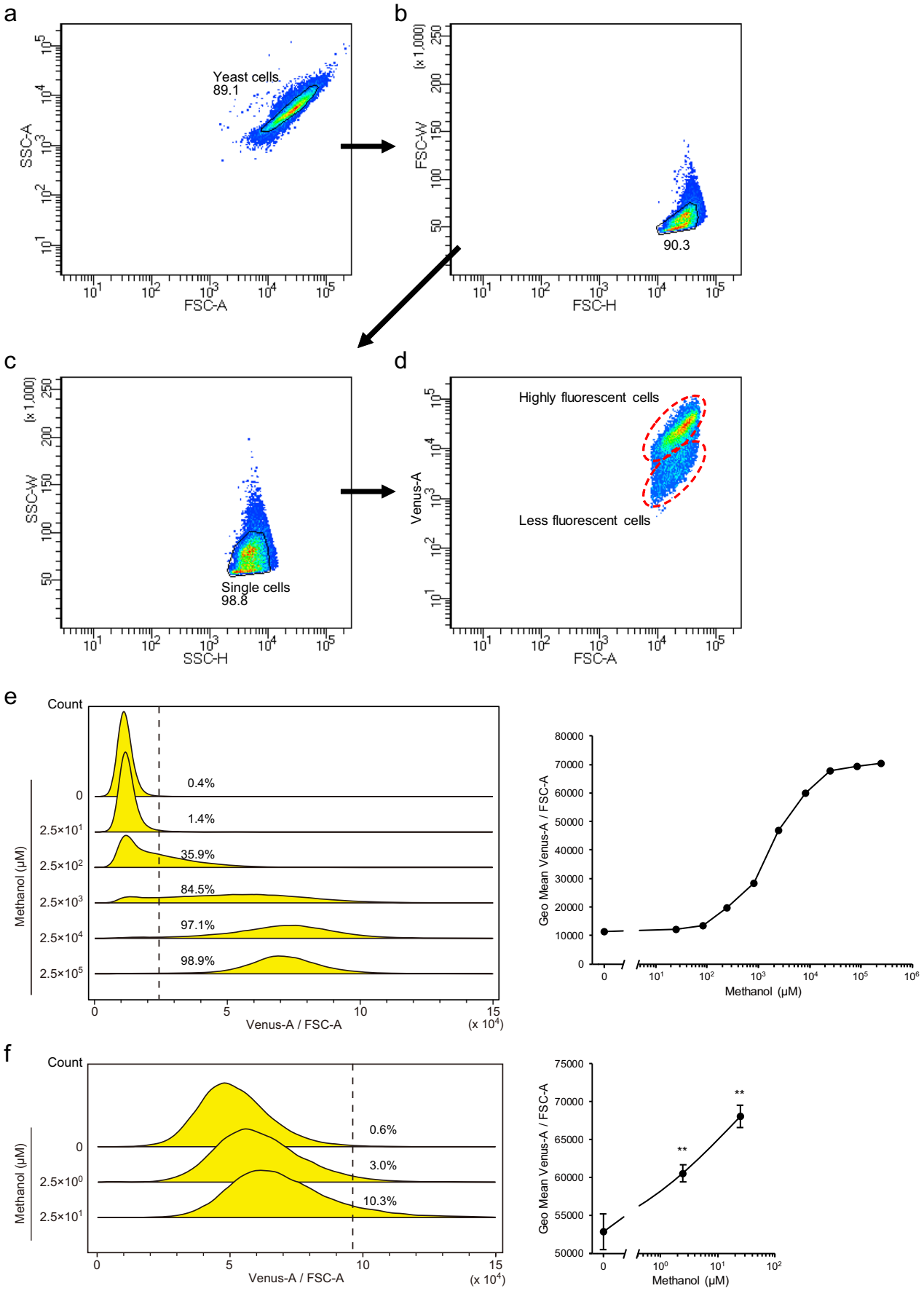
Finally, we conducted a time-course analysis of the methanol sensor cells to optimize the incubation time prior to observation. The methanol sensor cells were incubated in methanol medium and observed at 2-h intervals for 6 h. The cellular fluorescence intensity with 0–2.5×10^2 μM methanol at 2 h was almost the same as that at 0 h, while the cellular fluorescence intensity at 2.5×10^3 μM methanol was significantly higher than that without methanol (Fig. 2e, $p < 0.05$). The cellular fluorescence intensity with 25 μM methanol at 4 h was significantly higher than that without methanol ($p < 0.01$). The cellular fluorescence intensity without methanol at 6 h was distinguishable only from that at 2.5×10^3 μM methanol ($p < 0.05$).

Analysis of distribution and dose response of the cellular fluorescence intensity

Cellular adaptation to methanol is not necessarily homogenous in the population. We applied FACS analysis to examine the distribution of cellular fluorescence intensity in response to methanol dose to determine if individual cells with high fluorescence intensity are indeed sensing a high dose of methanol.

First, single yeast cells were separated from dividing cells, multiple cells and other particles through a gating strategy based on the forward and side scatter (Fig. 3a–c, cf. Fig. 3 legends for details). At least 30,000 cells were analyzed per sample. We found that Venus fluorescence was proportional to

Fig. 3 Distribution and dose-response of the cellular fluorescence intensity of methanol sensor cells, revealed by FACS analyses. Cells were precultured on glucose medium to late-exponential phase and transferred to methanol medium and analyzed after 4 h of incubation. Single cells were selected using the gates from **a** to **c** in order. **a** Yeast cells were selected on the basis of the forward scatter area (FSC-A) and the side scatter area (SSC-A). **b** First selection of single cells on the basis of the forward scatter height (FSC-H) and the width (FSC-W). **c** Second selection of single cells on the basis of the side scatter height (SSC-H) and width (SSC-W). **d** Plot of the Venus fluorescence area (Venus-A), detected at a PMT voltage of 700, against FSC-A of the single cells. The Venus-A of highly fluorescent cells and less fluorescent cells were both proportional to each FSC-A. Hereafter, the Venus fluorescence normalized to the forward scatter (Venus-A/FSC-A value) was used to represent the fluorescence intensity of the single cells. **e**, **f** Distribution and geometric mean of the Venus-A/FSC-A values at various concentrations of methanol measured at a Venus PMT voltage of 700 (**e**) or 900 (**f**). At least 30,000 cells were measured to generate each histogram and calculate each geometric mean. The maximum level of the Venus-A/FSC-A value without methanol is defined as the value indicated by dashed lines on the histograms. Percentages of cells with Venus-A/FSC-A values above the maximum level without methanol are indicated on the histograms. For **f**, the histograms represent typical data from three independent experiments starting from three distinct single colonies, followed by transfer to methanol medium in triplicate. Data from triplicates were averaged for each independent experiment, and shown on the plot. Error bars represent means ± s.d. of three independent experiments. Statistical significance against the data at 0 μM methanol was calculated by a two-tailed paired *t* test (**$p < 0.01$)


a
Yeast cells
89.1
SSC-A
FSC-A
b
90.3
FSC-W
FSC-H
c
Single cells
98.8
SSC-W
SSC-H
d
Highly fluorescent cells
Less fluorescent cells
Venus-A
FSC-A
e
Count
0.4%
1.4%
35.9%
84.5%
97.1%
98.9%
Methanol (μM)
Venus-A / FSC-A
Geo Mean Venus-A / FSC-A
Methanol (μM)
f
Count
0.6%
3.0%
10.3%
Methanol (μM)
Venus-A / FSC-A
Geo Mean Venus-A / FSC-A
Methanol (μM)

the forward scatter, which reflects cell size (Fig. 3d). Thus, Venus fluorescence normalized to the forward scatter (Venus-A/FSC-A) was considered to represent the fluorescence intensity of a single cell.

Next, we analyzed the distribution of the Venus-A/FSC-A value of the sensor cells induced by various methanol concentrations. Virtually all cells showed a basal level of Venus-A/FSC-A without methanol (Fig. 3e, left). As the methanol concentration increased, we observed increasing numbers of cells with Venus-A/FSC-A values above the maximum level without methanol. Thus, the cellular response to methanol was not uniform in the population; nevertheless, the number of cells with an increased Venus-A/FSC-A value was positively correlated with the methanol dose. In addition, the mode and the tail of the distribution shifted to higher Venus-A/FSC-A values as the methanol concentration increased up to 2.5×10^4 μM. For quantitative evaluation of the dose-response of the population, we used the geometric mean of Venus-A/FSC-A values, and found that this value increased up to 2.5×10^5 μM methanol (Fig. 3e, right).

To examine the cellular fluorescence intensity at low concentrations of methanol, we gained sensitivity of fluorescence detection by increasing the PMT voltage for Venus fluorescence. We found that the mode and the tail of the distribution of the Venus-A/FSC-A value shifted higher even at 2.5 μM methanol (Fig. 3f, left), showing a detection limit of 2.5 μM (Fig. 3f (right), $p < 0.01$). Thus, FACS analysis of the optimized methanol sensor resulted in the detection of methanol at micromolar concentrations (Fig. 3e, f).

Application of the methanol sensor for the detection of methanol-producing heterologous PME activity

The enzyme assay conditions were established as follows. First, the methanol sensor cells harboring the gene encoding a methanol-producing enzyme under the control of the constitutive *GAP* promoter (P_{GAP}) were precultured in the glucose medium. At this stage, the enzyme is highly produced, but expression of the Venus-PTS1 gene under the control of P_{DAS2} is repressed. The cells were then transferred into the enzyme assay medium containing the substrate. Methanol produced from the substrate and its metabolism induce Venus-PTS1 during 4 h of incubation (Fig. 4a).

The *A. niger* PME, an extracellular enzyme that hydrolyzes methyl-esterified pectin to polygalacturonate and methanol, was selected as the model methanol-producing enzyme. We constructed expression vectors for the *A. niger* PME tagged with 6xHis at the C-terminus under the control of P_{GAP}, and introduced them into the methanol sensor strain. We obtained single- and two-copy strains as revealed by Southern blot analysis (Fig. S2). Next, these strains were grown on glucose medium to late-exponential phase, transferred to PME assay medium, and incubated for 4 h. During this period, PME-His_6 synthesized before and/or after transfer is secreted into the PME assay medium. The cell density in the PME assay medium was set below an OD_{610} of 0.1 to prevent exhaustion of the substrate, i.e., methylester groups of pectin. According to FACS analysis, cells expressing PME-His_6 showed a cell density-dependent increase in the geometric mean of the Venus-A/FSC-A value (Fig. 4b). We noted that the host sensor cells also showed a relatively moderate increase in the geometric mean of the Venus-A/FSC-A value, which was ascribed to the endogenous PME activity (Kawaguchi et al. 2014; Nakagawa et al. 2005). The two-copy strain showed a higher geometric mean of the Venus-A/FSC-A value than cells containing a single copy. In addition, the mode and the tail of the distribution of the Venus-A/FSC-A value increased with increasing amounts of enzyme, i.e., the cell density or the copy number (Fig. S3). Methanol-sensing cells gave significant fluorescence not only in response to methanol but also formaldehyde and formate, which are downstream metabolites of methanol; no fluorescence increase was detected with methane (another C1 compound) or polygalacturonate (another product of PME catalysis). Therefore, the Venus-A/FSC-A value was assumed to represent pectin-dependent methanol formation.

We next biochemically confirmed the production of PME-His_6 in these strains by immunoblot analysis. In both PME-containing strains, we detected broad bands of 50 to 60 kDa that were attributed to PME-His_6 (Fig. 4c). Although this band size disagrees with the estimated value from the amino acid sequence of the processed form of PME-His_6, glycosylation via the secretion pathway was assumed to result in the increased molecular weight. The band of PME-His_6 from the two-copy strain was more intense than that from the single-copy strain; the two-copy strain produced ca. 1.5 times more PME-His_6 (Fig. 4d). In addition, PME enzyme activity in the broth of the two-copy strain was about 1.8 times greater than that of the single-copy strain (Table 3), which is consistent with the production level of PME-His_6. Hence, the PME-His_6 gene copy number reflected the PME-His_6 production and activity level. From these results, we concluded that methanol sensing by single cells was successful for evaluating the activity of a heterologously expressed methanol-producing enzyme, and could discriminate the 1.5-fold difference in PME activity between the two strains.

Discussion

In this study, we developed a *P. pastoris* single-cell methanol sensor that can be applied to evaluate the activity of methanol-producing enzymes. The detection limit for methanol was improved by selection of the methanol-inducible promoter and the genetic background, and optimization of the assay conditions. Application of FACS enabled us to analyze

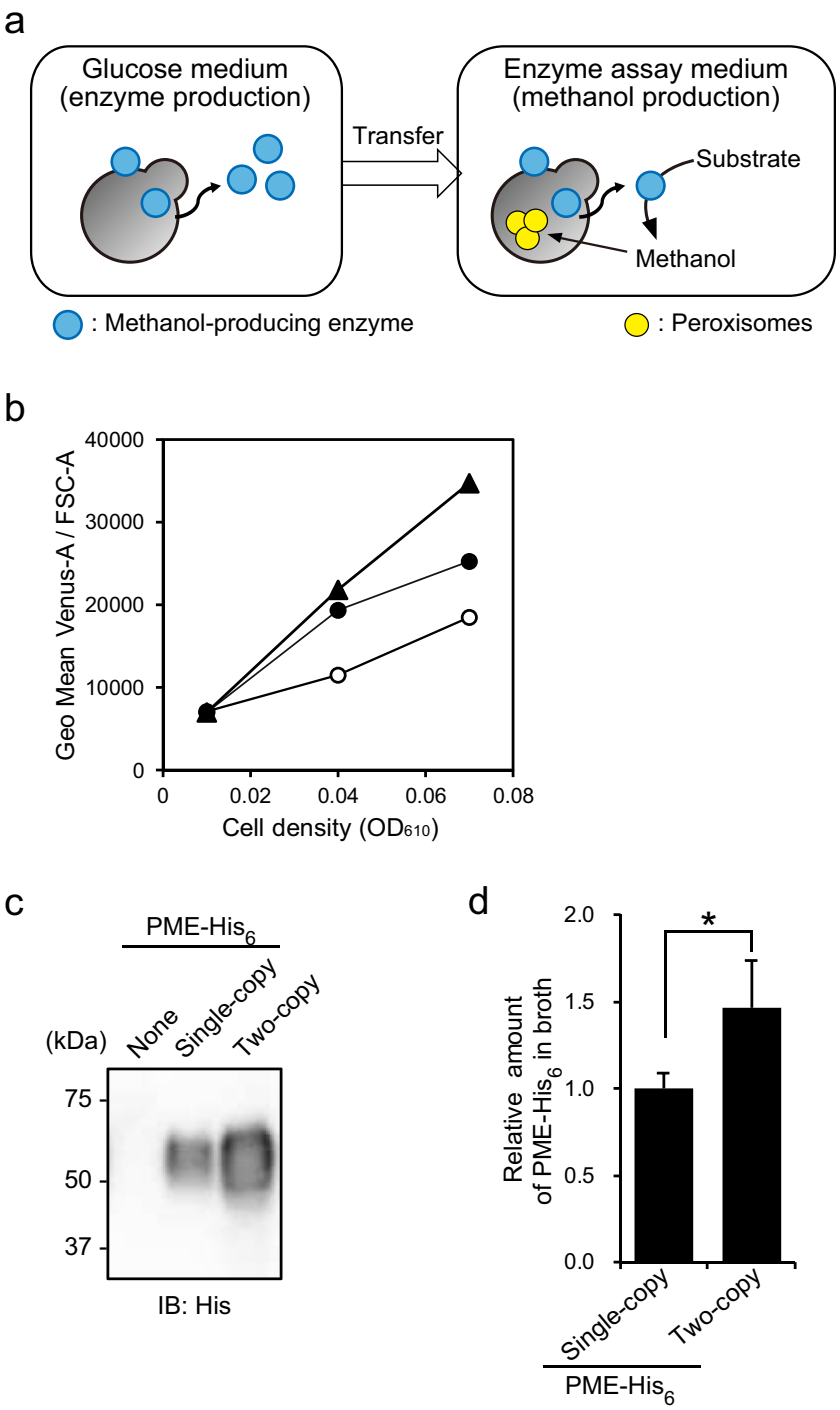


Fig. 4 Production of His_6-tagged *A. niger* PME in the methanol sensor host. **a** Schematic illustration of methanol sensor-based evaluation of PME activity. Cells from glucose medium were washed and transferred to PME assay medium. Since PME-His_6 is expressed from a constitutive promoter, PME-His_6 synthesized before and/or after medium shift is secreted into the PME assay medium. Secreted PME-His_6 hydrolyzes the methylester group of pectin, producing methanol, which is expected to increase the cellular fluorescence intensity. **b** FACS analysis of the host methanol sensor cells (open circles), the cells harboring one and two copies of PME-His_6 (closed circles and closed triangles, respectively) incubated in PME assay medium. Cells were precultured in glucose medium to the late-exponential phase, transferred to PME assay medium, and incubated for 4 h. FACS analysis was performed as in Fig. 3e. **c** Secretion of PME-His_6 detected by immunoblotting. Cells were grown in glucose medium for 30 h, and samples prepared from equal volumes of supernatant were loaded in each lane. **d** Densitometry of three biological replicates of immunoblot analyses as in **c**. For **d**, error bars represent means ± s.d. of samples from three independent cultures. Statistical significance was calculated by a two-tailed Student's *t* test assuming equal variance (n.s., $p > 0.05$; $^*p < 0.05$)

Table 3 PME enzyme activities in culture broth of the methanol sensor and transformant strains

PME expression	None	Single-copy	Two-copy
Activity[a] (U/mL broth)	n.d.[b]	16.2 ± 4.6	29.7 ± 0.4

[a] Means ± s.d. of three measurements

[b] Not detected ($< 7.41 \times 10^{-2}$ U/mL broth)

fluorescence from ~30,000 single cells, >100 times more than with fluorescence microscopy. To our knowledge, this is the first report of the distribution of methanol-induced protein production at a single-cell level. Through these analyses, we revealed the relationship between the distribution of cellular fluorescence and methanol dose, and found that the distributions of cellular fluorescence in the presence and absence of micromolar amounts methanol were clearly distinguishable. The detection limit of 2.5 μM is 100-fold lower than that of

a similar fluorescent microscopy-based *C. boidinii* methanol sensor (Kawaguchi et al. 2011), and comparable to that of gas chromatography (Pontes et al. 2009).

This single-cell sensor was used as the expression host for the heterologous methanol-producing enzyme, PME. The detection limit of the present methanol sensor was confirmed to be sufficient for evaluation of the produced enzyme activity. It is unlikely that the methanol concentration rose above the detection limit soon after the start of incubation of sensor cells with the substrate. We assume that the methanol sensor cells not only responded to the methanol concentration at the start of the enzyme reaction, but also monitored further methanol metabolism for induction and synthesis of the Venus-PTS1 protein, because *aox1*Δ cells impaired in methanol metabolism showed lower cellular fluorescence intensity than the wild-type cells. Indeed, not only methanol but also the downstream metabolites formaldehyde and formate induced fluorescence from methanol-sensor cells (Sakai et al. 1999) (data not shown). These properties are ideal for the detection of very weak methanol-producing enzyme activity in the methylotrophic yeast *P. pastoris*.

Since the level of cellular enzyme activity correlated with the maximum level of cellular fluorescence, highly active cells could be selected and isolated by FACS. Indeed, the mixture of single-copy and two-copy PME-expressing cells was separated by FACS (Fig. S4). Such screening cannot be achieved by a methanol sensor constructed with microbial cells immobilized on an oxygen electrode (Wen et al. 2014). This study demonstrates the feasibility of a high-throughput screen for cells with high methanol-producing enzyme activity.

The methanol-inducible promoter affected the detection limit of the methanol sensor cells. In the presence of glucose, methanol-inducible genes are completely repressed (Tschopp et al. 1987). In the absence of glucose or other repressing carbon sources, the expression level is elevated regardless of the presence of methanol. This methanol-independent gene activation is referred to as "de-repression." On the other hand, methanol-dependent gene activation is called methanol induction, and results in a greater gene expression level than de-repression (Yurimoto et al. 2011). The desired characteristics of the promoter in the methanol sensor are a low level of de-repression and high level of methanol induction. The levels of de-repression and methanol induction differ among methanol-inducible promoters (Sakai et al. 1996; Vogl et al. 2016; Yurimoto et al. 2000). The level of de-repression with the alcohol oxidase (AOX)-encoding gene promoter was reported to be higher than that of the dihydroxyacetone synthase (DAS)-encoding gene promoter, while the level of methanol induction of the DAS-encoding gene promoter was higher than that of the AOX-encoding gene promoter (Tschopp et al. 1987; Yurimoto et al. 2000). Also, the DAS-encoding gene is known to be fully activated at an earlier stage during methanol induction than the AOX-encoding gene in another methylotrophic yeast, *C. boidinii* (Sakai et al. 1996). Our present results confirmed the superiority of the *DAS2* promoter over the *AOX1* promoter for use in the methanol sensor.

The physiological state of sensor cells was also found to be important for the detection of low concentrations of methanol. Release from glucose repression is a prerequisite for methanol induction (Yurimoto et al. 2000; Yurimoto et al. 2011). During preculturing on glucose medium, glucose completely represses methanol-inducible gene expression until the mid-exponential phase. However, glucose repression is gradually attenuated as glucose is consumed during growth, so late-exponential phase cells are assumed to be more de-repressed than mid-exponential phase cells (Fig. 2a, b). Therefore, the physiological state of late-exponential phase cells is suitable for a sensitive response to low concentrations of methanol.

The methanol-sensing system developed in this study will play a key role in high-throughput screening for cells harboring high methanol-producing enzyme activity, and thereby, engineering of an enzyme mutant with high specific activity and/or stability. One important substrate for this system would be methane. Since functional expression of methane monooxygenase in heterologous hosts has not been successful (Clomburg et al. 2017), our system can facilitate the selection of the cells that express a functional enzyme.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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