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Dr. Xiaoyan Cao
Review Editor
Journal of Visual Experiments

Dear Dr. Cao:

I wish to submit the revised version of our manuscript (ID: JoVE61219), presently titled as "Direct liquid-culture screening method for evaluating the production of heterologous proteins using an auxotrophic mutant of *Aspergillus oryzae*" in view of your decision letter dated February 12, 2020.

We are thankful for a thorough review of our work and for the constructive criticisms. The comments made by you and the reviewers have helped us improve our manuscript. We have revised the manuscript considering all the suggestions. The point-by-point response to all the comments is being submitted herewith.

We hope that the revised manuscript addresses all the concerns that were raised and would be suitable for publication in this prestigious journal.

Thank you for your consideration. I look forward to hearing from you.

Sincerely,
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TITLE:

Direct Liquid-Culture Screening for Evaluating the Production of Heterologous Proteins Using an Auxotrophic Mutant of *Aspergillus Oryzae*

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KEYWORDS:

Aspergillus oryzae, uridine auxotroph, gene introduction, polyethylene glycol (PEG) transformation, protoplast, liquid culture, high-throughput screening, microplate

SUMMARY:

A direct liquid-culture (DLC) screening method has been developed which significantly reduces the time required for polyethylene glycol (PEG)-mediated protoplast transformation of the filamentous fungus, *Aspergillus oryzae*, when employed for evaluation of the secretory production of heterologous proteins. This method dramatically increases the throughput of the evaluation protocol.

ABSTRACT:

Aspergillus oryzae, a filamentous fungus, is one of the most widely used hosts for industrial applications including large-scale production of proteins. A polyethylene glycol (PEG)-mediated protoplast transformation method is generally used for the introduction of heterologous genes into *A. oryzae*. The conventional method typically requires three weeks for the screening of favorable transformants. Here, a new technique, the direct liquid-culture (DLC) screening method, is introduced which reduces the screening time to six days in a 200 mL flask format or to 10 days in a 24 well microplate format. The DLC screening method ensures the acquisition of positive transformants and evaluation of the secretory production of heterologous proteins in a single step, unlike the conventional screening method where two separate steps are required for the same. The protocol for PEG-mediated protoplast transformation of *A. oryzae* is described, which consists of five steps: preparation of fresh spore suspension, preculture, preparation of protoplasts, introduction of DNA, and DLC screening. For successful results in DLC screening, it is critical to use a nutrient-rich medium with optimized osmotic pressure. The protocol should further popularize the use of *A. oryzae* as a host of choice in the industrial production of proteins.

INTRODUCTION:

Aspergillus oryzae is an important microorganism in the Japanese food industry that has been used for over 1,000 years in the production of fermented foods, such as *sake* (rice wine), *shoyu* (soy sauce), and *miso* (soybean paste)[1,2]. It has the ability to secrete a large amount of proteins, such as proteases and amylases[3]. Genome sequence information for *A. oryzae* is also available[4]. Moreover, powerful and practically useful genetic engineering techniques have been established for this fungus[5–10]. Favorable transformants have been used as hosts for secretory production of heterologous proteins[11-14].

Electroporation, Agrobacterium-mediated transformation, and polyethylene glycol (PEG)-mediated protoplast transformation are the techniques used for introducing heterologous genes into *A. oryzae*[15–17]. The PEG-mediated protoplast transformation method has been widely used since it was first reported for *Neurospora crassa* in 1979[18]. In this method, protoplasts are prepared and mixed with PEG and the heterologous gene that is to be introduced into the cells. The walls of protoplasts are enzymatically compromised, which makes the cells vulnerable to physical stress and changes in osmotic pressure[19,20]. The conventional screening method employed in the secretory production of heterologous proteins includes three steps: acquisition of positive transformants (on soft agar plate), selection of true and false transformants (on agar plate), and evaluation of the secretory production of heterologous proteins (in liquid culture); each of these steps takes seven days (**Figure 1**). Thus, the conventional method typically requires about three weeks.

The time required for screening of transformed *A. oryzae* cells is much longer than that required for other microorganisms commonly used in biotechnology research, making the process more cumbersome. For example, when using *Escherichia coli* as a host, it takes approximately two days from the introduction of DNA to confirmation of its effect[21].

To circumvent the limitation associated with the use of *A. oryzae* as mentioned above, herein, a new direct liquid-culture (DLC) screening method is introduced, which enables a more rapid and simple screening for evaluation of the secretory production of heterologous proteins (**Figure 1**). In the DLC method, a uridine auxotrophic mutant and a nutrient-rich liquid medium are used. Using this method, the screening step can be completed within six days after the introduction of DNA into the protoplasts in a 200 mL flask format or within 10 days in a 24 well microplate format. Furthermore, the time-consuming and laborious preparation of agar plate media is not needed in this method. There is a huge advantage in using the newly described method, especially considering the fact that the conventional method requires two different media: the soft agar plate for acquisition of positive transformants, which requires careful handling and temperature control, and solid agar plate for selection of true transformants.

[Place **Figure 1** here]

PROTOCOL:

1. Preparation of fresh spore suspension

1.1. Inoculate 20 µL of a stock spore suspension (1 x 10^7 spores/mL) in the center of a culture plate containing Czapek-Dox (CD) medium with 20 mM uridine (autoclaved at 121 °C for 20 min, **Table 1**).

1.2. Incubate at 30 °C for 7 days to promote spore formation.

1.3. Add 1.5 mL of 0.01 % Tween 20 solution (autoclaved at 121 °C for 20 min) to the culture plate containing spores and suspend the spores by scraping with a cell spreader.

1.4. Collect the spore suspension in a sterile 1.5 mL microcentrifuge tube using a pipette.

1.5. Store the suspension at 4 °C until use.

NOTE: Use the spore suspension in the following procedure within 2 weeks. To make a stock for long-term storage, add glycerol to the spore suspension to a final concentration of 15% and store at -80 °C.

2. Preculture

2.1. Add 100 mL of polypeptone-dextrin (PD) medium (**Table 2**) in a 500 mL Erlenmeyer flask, and autoclave it at 121 °C for 20 min.

2.2. After confirming that the temperature of the medium is reduced, add the uridine solution, sterilized by passing through a filter with a pore size of 0.22 µm, to a final concentration of 20 mM.

2.3. Add 200 µL of the spore suspension (1 x 10^7 spores/mL) prepared in step 1.4, and incubate at 30 °C with shaking at 120 rpm for 36 h.

3. Preparation of protoplasts

3.1. Harvest the fungal biomass prepared in step 2.3 on a glass filter with pore size of 30 µm (autoclaved at 121 °C for 20 min).

3.2. Pour 100 mL of distilled water (DW) (autoclaved at 121 °C for 20 min) on the glass filter and stir several times with a spatula (autoclaved at 121 °C for 20 min).

3.3. After washing with DW, pour 100 mL of sodium chloride (NaCl) buffer (autoclaved at 121 °C for 20 min, **Table 3**) on the glass filter and stir several times with a spatula.

3.4. Transfer about 1−2 mL (wet volume) of the cells to a 50 mL conical tube using a spatula (autoclaved at 121 °C for 20 min).

3.5. After adding 15 mL of the enzyme solution (sterilized by passing through a 0.22 µm pore

sized filter, **Table 4**) to a 50 mL conical tube, add NaCl buffer until the total volume reaches 30 mL.

3.6. Close the lid and seal with paraffin film. Incubate at 30 °C with shaking at 60 rpm for 2 h.

3.7. Flow the resulting solution through a 70 µm cell strainer attached to the 50 mL conical tube to remove the unreacted mycelia.

3.8. Centrifuge the protoplast solution in a 50 mL conical tube at 2,150 x *g* and 4 °C for 20 min.

3.9. Gently discard the supernatant to prevent pellet from dislodging. Add 1 mL of ice-cold sterilized solution B (autoclaved at 121 °C for 20 min, **Table 5**) and gently suspend the precipitated protoplasts by pipetting.

3.10. Transfer the suspended protoplasts to a sterile 1.5 mL tube.

3.11. Centrifuge at 2,220 x *g* and 4 °C for 5 min and remove the supernatant. Then, add 1 mL of ice-cold solution B and gently suspend the pellet by pipetting.

3.12. Repeat step 3.9.

3.13. Using a hemocytometer, measure the number of protoplasts under a microscope.

3.14. Prepare a protoplast suspension (1−3 x 10^7 protoplasts/mL) in solution B and store it on ice until use.

NOTE: Use the protoplast suspension immediately in the following procedure.

4. Introduction of DNA for secretory production of protein

4.1. Using a plasmid containing a DNA cassette for secretory production of protein as a template, amplify the DNA fragment required for secretory production by PCR.

4.2. Prepare the DNA sample to be used for transformation by processing the PCR product using a PCR purification kit.

4.3. Add 1 mL of the protoplast solution, 200 µL of solution C (autoclaved at 121 °C for 20 min, **Table 6**), and 50 µL of the DNA sample (0.5−1 µg/µL) in a sterile, precooled 15 mL conical tube, and mix the solution gently by pipetting.

NOTE: Solution C is highly viscous and can be difficult to handle; ensure thorough mixing by pipetting.

4.4. Incubate the tube on ice for 30 min.

4.5. Add 1.5 mL of solution C, mix gently by pipetting, and then, leave the solution at room temperature for 20−30 min.

5. Direct liquid culture screening

NOTE: Select the culture system using Erlenmeyer flasks (section 5.1) or microplates (section 5.2).

5.1. Culture system using a 200 mL Erlenmeyer flask

5.1.1. Add 50 mL of PD medium containing 0.8 M sorbitol to a 200 mL Erlenmeyer flask, and sterilize by autoclaving at 121 °C for 20 min.

5.1.2. After confirming that the temperature of the medium is reduced, add the protoplast suspension prepared in step 4.5 to a final concentration of 1 x 10^5 protoplasts/mL.

5.1.3. Incubate the culture for 6 days at 30 °C with shaking at 120 rpm.

5.1.4. Collect 1 mL of the medium and store in a 1.5 mL tube at 4 °C. Store the culture sample at -20 ° C, if not analyzed immediately.

5.1.5. Mix 20 µL of culture sample and 20 µL of sodium dodecyl sulfate (SDS) sample buffer (**Table 7**) in a 1.5 mL tube and boil at 95 ° C for 5 min.

5.1.6. After cooling on ice, load 20 µL of the boiled sample and 5 µL of prestained protein standard on a precast gel and analyze the secretory production of the target heterologous protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[10].

5.2. Culture system using a 24 well microplate

5.2.1. Add the protoplast suspension prepared in step 4.5 to sterilized 0.8 M sorbitol-containing PD medium to a final concentration of 1 x 10^5 protoplasts/mL, and gently mix by pipetting.

5.2.2. Add 1 mL of the solution prepared in step 5.2.1 to three wells in a 24 well microplate.

5.2.3. Affix the lid and incubate at 30 °C with shaking at 175 rpm for 10 days.

5.2.4. Collect 1 mL of medium and store in a 1.5 mL tube at 4 ° C. Store the culture sample at -20 ° C, if not analyzed immediately.

5.2.5. Mix 20 µL of the culture sample and 20 µL of SDS sample buffer in a 1.5 mL tube and boil at 95 ° C for 5 min.

5.2.6. After cooling on ice, load 20 µL of the boiled sample and 5 µL of prestained protein standard on a precast gel and analyze the secretory production of the target heterologous protein by SDS-PAGE[10].

REPRESENTATIVE RESULTS:

The results for the introduction of the DNA expression cassette coding for *Talaromyces cellulolyticus* cellobiohydrolase (CBH: GenBank Accession Number E39854) into a uridine auxotroph of *A. oryzae* strain HO4[22] and screening for the secretory production of the heterologous protein are described below.

Preparation of fresh spore suspension

The final yield of spore suspension from one agar plate was 1 mL (1 x 10^7 spores/mL).

Preculture

After incubation for 36 h, ~5 mL (wet volume) of the fungal biomass was obtained from one flask when harvested at step 3.1. The yield sufficed for at least two trials of step 3.2.

Preparation of protoplasts

As the enzymatic reaction proceeded, the aggregated mycelia dissolved and the solution became cloudy (**Figure 2**), indicating the formation of protoplasts.

Introduction of DNA for secretory production of protein

The DNA cassette for secretory production of CBH was amplified by PCR using the plasmid pPPAe8-CBH1 as a template[22]. The molecular weight of CBH calculated from the amino acid sequence excluding the putative signal peptide is 52 kDa.

Culture system using a 200 mL Erlenmeyer flask

Protoplasts mixed with the DNA were added to PD medium containing 0.8 M sorbitol as well as to medium containing 0 and 1.6 M sorbitol (suboptimal levels). After incubation for 6 days, prolific cell growth was observed in the medium with 0.8 M sorbitol, whereas the growth was less in the presence of 1.6 M sorbitol; no growth was observed in the absence of sorbitol (**Figure 3A**). The secretion of CBH in the supernatant of the culture with 0.8 M sorbitol was confirmed by SDS-PAGE[10] (**Figure 3B**). As a negative control, 200 µL of a spore suspension (1 x 10^7 spores/mL) of the HO4 strain was added to 100 mL of PD medium, and the supernatant from a sample cultured for 6 days was analyzed by SDS-PAGE[10]. As shown in **Figure 3B**, a band of the same size as reported previously using the same *cbh* gene was observed[11].

Culture system using a 24 well microplate

Protoplasts that were mixed with DNA and those that were not mixed were added to PD medium containing 0.8 M sorbitol and cultured for 10 days (**Figure 4A**). The wells 1−3 contained samples in which DNA was mixed. The well 4 contained a sample in which DNA was not mixed (negative control). On day 6, growth was observed in all the 4 wells. On day 10, an increase in the amount of mycelium was observed in wells 1 and 2 relative to the growth on day 6, whereas growth in wells 3 and 4 appeared to have ceased after day 6. From these results, it is

predicted that wells 1 and 2 had the true transformants and well 3 was a false positive case. The secretion of CBH in the supernatant collected from wells 1, 2, and 4 was confirmed by SDS-PAGE[10] (**Figure 4B**). As shown in **Figure 4B**, a band of the same size as reported previously using the same *cbh* gene was confirmed[11].

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of polyethylene glycol (PEG)-mediated protoplast transformation of the filamentous fungus, *Aspergillus oryzae*. (**Top panel**) Conventional screening method. (**Bottom panel**) Direct liquid-culture (DLC) screening method.

Figure 2: Picture showing protoplast suspension prepared in protocol section 3. (**A**) Before and (**B**) after the enzymatic treatment.

Figure 3: Direct liquid-culture (DLC) screening of *Aspergillus oryzae* strain HO4 transformed with an expression cassette of *Talaromyces cellulolyticus* cellobiohydrolase (CBH) in a 200 mL Erlenmeyer flask. (**A**) Comparison of growth in culture medium containing 0, 0.8, and 1.6 M sorbitol after incubation for 6 days. (**B**) Confirmation of CBH secretion in the supernatant of the culture with 0.8 M sorbitol by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. M: Marker; 1: Culture supernatant of non-transformed *A. oryzae* strain HO4; 2: Culture supernatant (0.8 M sorbitol) of transformed *A. oryzae* strain HO4. Red rectangle shows the target heterologous protein. Supernatant on day 6 of culture was used for the assay.

Figure 4: Direct liquid-culture (DLC) screening of *Aspergillus oryzae* strain HO4 transformed with an expression cassette of *Talaromyces cellulolyticus* cellobiohydrolase (CBH) in a 24 well microplate. (**A**) Comparison of growth in culture medium containing 0.8 M sorbitol after incubation for 3, 6, and 10 days. Wells 1−3 contain samples mixed with DNA. Well 4 contains a sample to which DNA was not added (negative control). (**B**) Confirmation of CBH secretion in the supernatant of the culture with 0.8 M sorbitol by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. M: Marker; 1: Culture supernatant from well 1; 2: Culture supernatant from well 2; 4: Culture supernatant from well 4. Red rectangle shows the target heterologous protein. Supernatant on day 10 of culture was used for the assay.

Table 1: Composition of Czapek-Dox (CD) medium. Adjust the pH of the medium to 6 using 1 M NaOH and make the volume to 1 L with distilled water. After autoclaving, add uridine solution, sterilized by passing through a filter with a pore size of 0.22 µm, to a final concentration of 20 mM.

Table 2: Composition of polypeptone-dextrin (PD) medium. Adjust the pH of the medium to 6 using 1 M NaOH and make the volume to 1 L with distilled water. After autoclaving, add uridine solution, sterilized by passing through a filter with a pore size of 0.22 µm to a final concentration of 20 mM.

Table 3: Composition of NaCl buffer.

Table 4: Composition of the enzyme solution.

Table 5: Composition of solution B.

Table 6: Composition of solution C.

Table 7: Composition of SDS sample buffer.

DISCUSSION:

We have developed a system that allows the screening of *A. oryzae* transformants more rapidly than the conventional method, by conducting liquid culture of the protoplasts. The most critical aspect of this method is the osmotic pressure of the liquid medium. The osmotic pressure suitable for liquid culture was optimized using sorbitol. The growth of *A. oryzae* strain HO4 was most active in the presence of 0.8 M sorbitol (**Figure 3A**). In the conventional method using soft agar medium, an osmotic pressure of 0.5−1.2 M was reported to be suitable for *N. crassa*, *Aspergillus* sp., and *Trichoderma* sp.[21]. Osmotic pressure regulators include sorbitol, NaCl, potassium chloride (KCl), and magnesium sulfate ($MgSO_4$)[21]. Depending on the type of strain used, the osmotic pressure regulator and the target osmotic pressure may need to be adjusted.

It will be necessary to consider the composition of the enzyme solution for producing protoplasts when using other filamentous fungi as a host for secretory production of heterologous proteins. The composition used in this protocol was according to *Tamano* et al.[23]. *Endo* et al.[24] used a different enzyme solution without cellulase for *Aspergillus nidulans.*

In this protocol, PD medium is used in the screening method, which is suitable for the uridine-requiring strain. To establish an alternative protocol using an antibiotic or an auxotroph requiring a compound other than uridine for selection, the nutrient composition of the liquid medium must be optimized. For example, when using pyrithiamine for *A. oryzae*[25], it is necessary to use a medium that does not contain thiamine. Similarly, when using the *niaD* gene as an auxotrophic marker for *A. oryzae*[26], nitrate must be used as a single nitrogen source.

The volume and format of liquid culture affect the screening time. In the representative results, the 24 well microplate format required a longer time compared to the 200 mL Erlenmeyer flask format. This may be because of the differences (i) in the initial number of viable cells and (ii) in the dissolved oxygen content, which depends on the degree of aeration. In the 24 well microplate format, the selection between true and false transformants is easily achieved by visual inspection (**Figure 4A**).

In recent years, genetic modification using genome-editing technology has been reported in filamentous fungi[27,28]. The simultaneous introduction of two genes in a single transformation procedure using genome-editing technology has also been reported[29]. It may be possible to rapidly and simultaneously introduce multiple heterologous protein genes by combining genome-editing techniques and the DLC screening method using microplates. In this perspective, the merits ascribed to DLC should be much appreciated.

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DISCLOSURES:

The authors have nothing to disclose.

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441 *oryzae*. *Applied Environmental Microbiology*. **85** (3), e01896−e01918 (2019).

Figure 1

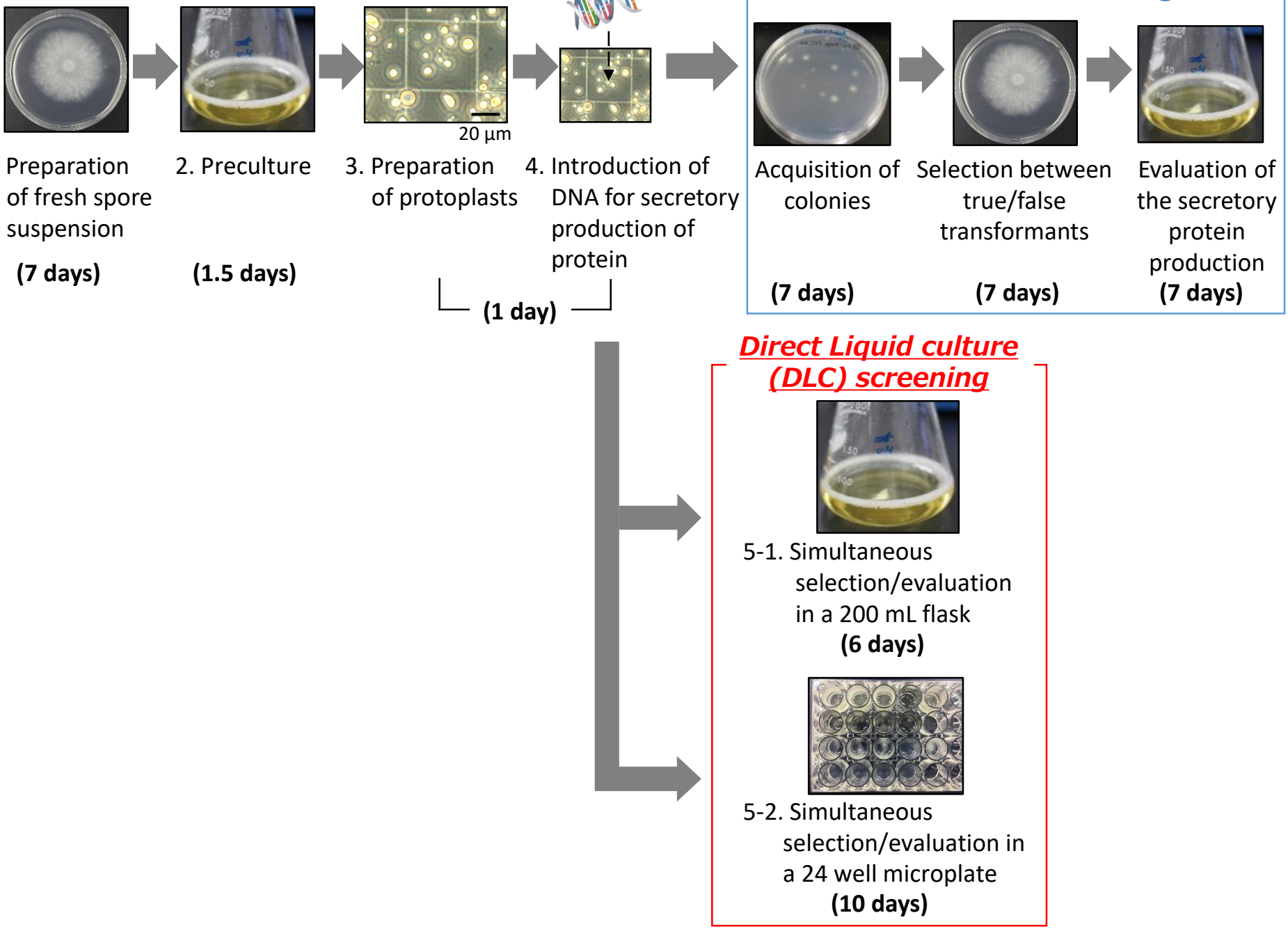


Figure 2

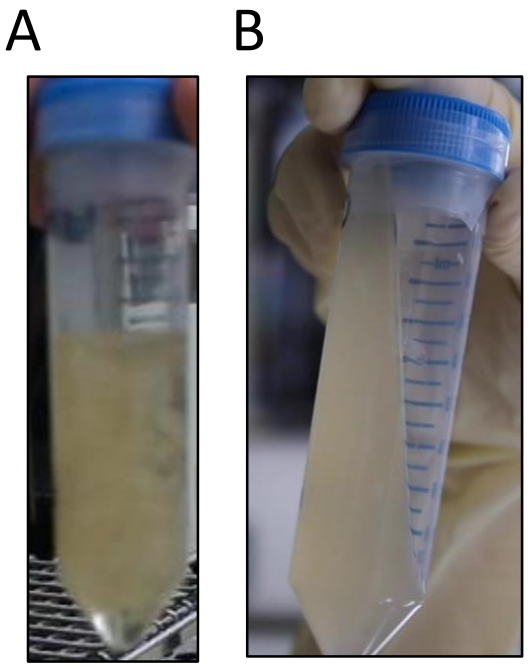
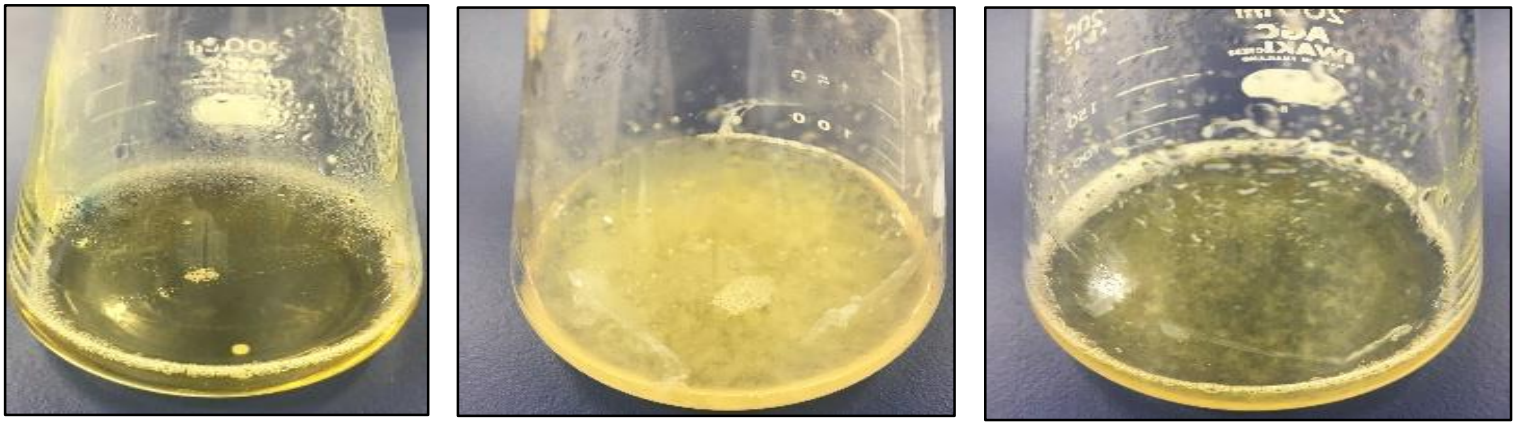


Figure 3

A



0 M sorbitol

0.8 M sorbitol

1.6 M sorbitol

B

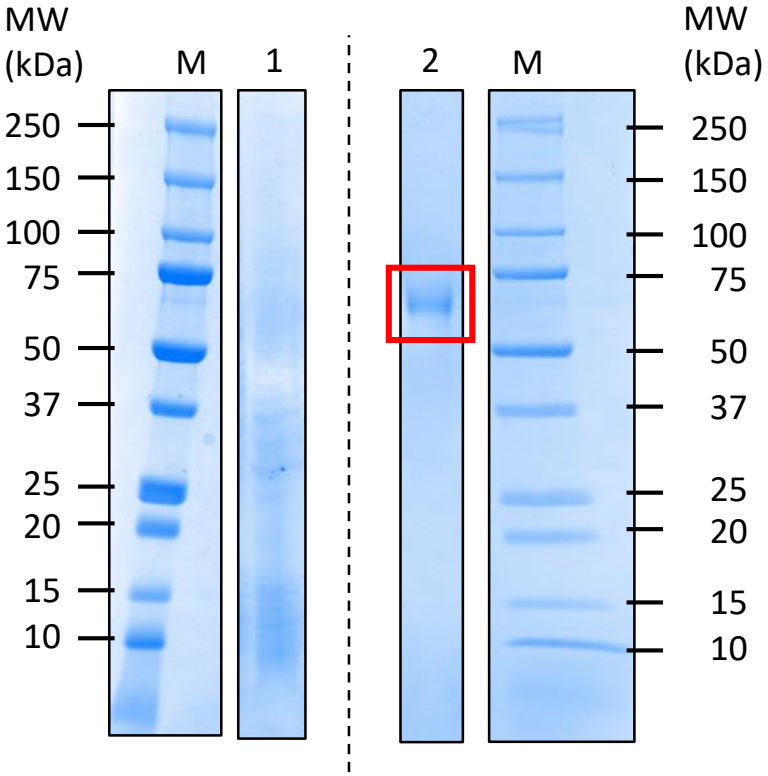


Figure 4

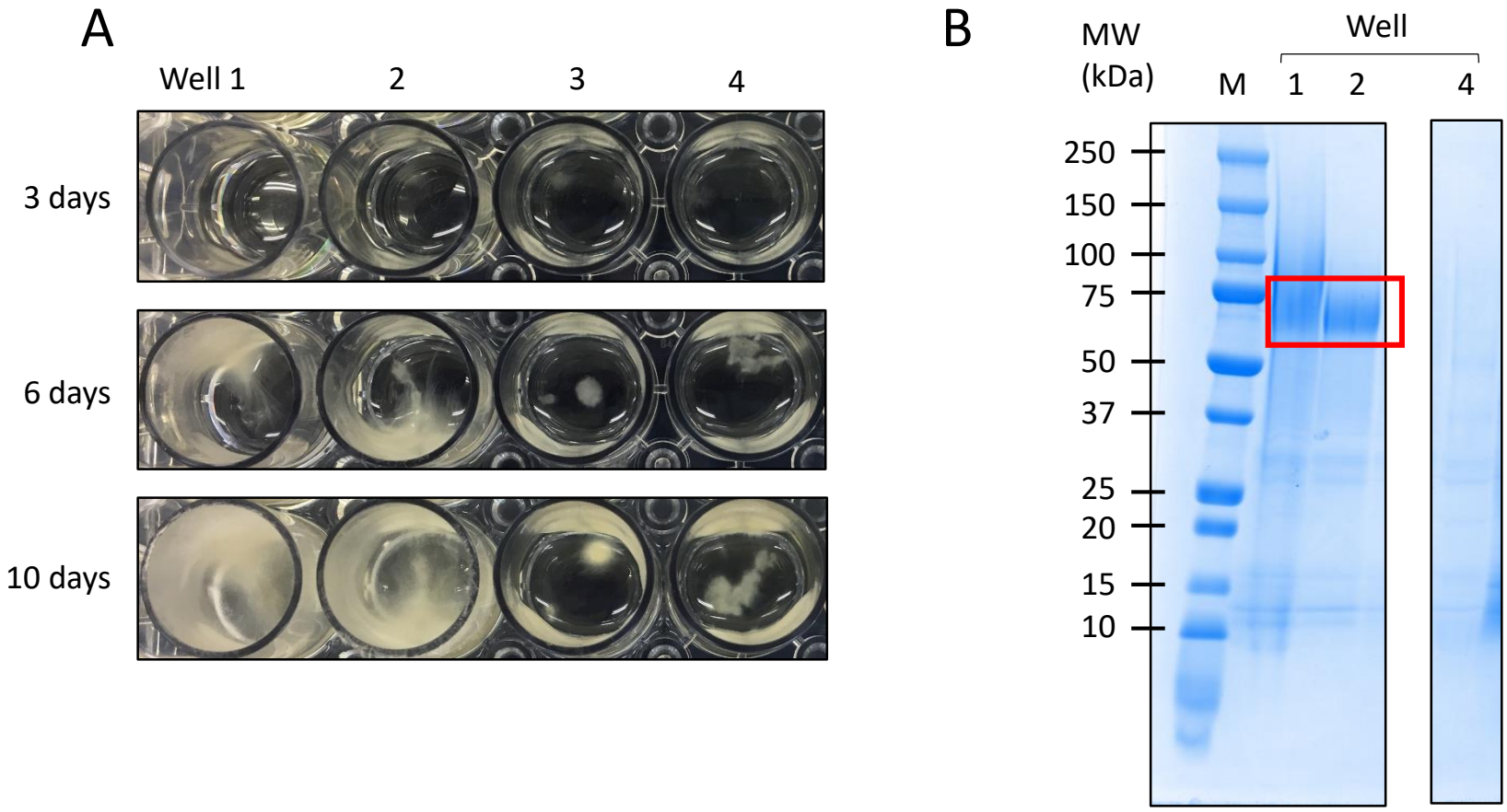


Table 1

Component	Amount (g) per liter medium
Dextrin hydrate	30
KCl	2
KH_2PO_4	1
$NaNO_3$	3
$MgSO_4 \cdot 7H_2O$	0.5
$FeSO_4 \cdot 7H_2O$	0.01
Agar	15

Table 2

Component	**Amount (g) per liter medium**
Dextrin hydrate	20
Polypeptone peptone	10
Casamino acids	1
KH_2PO_4	5
$NaNO_3$	1
$MgSO_4 \cdot 7H_2O$	0.5

Component	**Amount**
5 M NaCl	16 mL
1 M NaH_2PO_4	10 mL
Distilled water	Volume to 100 mL

Table 4

Component	Amount
Lytic enzyme	0.1 g
Yatalase	0.1 g
Cellulase R-10	0.05 g
NaCl buffer	Volume to 30 mL

Table 5

Component	**Amount**
D-Sorbitol	21.86 g
$CaCl_2$	0.555 g
1M Tris-HCl (pH 7.5)	1 mL
Distilled water	Volume to 100 mL

Table 6

Component	Amount
Polyethylene glycol	50 g
1 M Tris-HCl (pH 7.5)	1 mL
Distilled water	Volume to 100 mL

Component	Amount
2-Mercaptoethanol	2.5 mL
Sodium dodecyl sulfate	1 g
Sucrose	2.5 g
Bromophenol blue	1 mg
1 M Tris-HCl (pH 6.8)	6.25 mL
Distilled water	Volume to 25 mL

Name of Material/Equipment	Company	Catalog Number
1 M NaOH	NACALAI TESQUE, INC.	37421-05
1 M Tris-HCl (pH 7.5)	FUJIFILM Wako Pure Chemical Corporation	318-90225
1 M Tris-HCl (pH 6.8)	FUJIFILM Wako Pure Chemical Corporation	2106-100
1.5-mL Microcentrifuge tube	AS ONE Corporation	1-1600-03
15-mL Conical centrifuge tube	Becton, Dickinson and Company	352196
2-mercaptoethanol	Bio-Rad Laboratories, Inc.	1610710
24-well Microplate	AGC TECHNO GLASS CO., LTD.	3820-024
50-mL Conical centrifuge tube	Becton, Dickinson and Company	352070
70-µm Cell strainer	Becton, Dickinson and Company	352350
Agar	FUJIFILM Wako Pure Chemical Corporation	010-15815
Autoclave	TOMY SEIKO CO.,LTD.	LSX-700
Bromophenol blue	FUJIFILM Wako Pure Chemical Corporation	021-02911
$CaCl_2$	FUJIFILM Wako Pure Chemical Corporation	038-24985
Casamino acid	FUJIFILM Wako Pure Chemical Corporation	393-02145
Cellulase R-10	Cosmo Bio Co., Ltd.	16419
Dextrin hydrate	FUJIFILM Wako Pure Chemical Corporation	044-00585
D-Sorbitol	FUJIFILM Wako Pure Chemical Corporation	191-14735
e-PAGEL	ATTO CORPORATION	E-T/R1020L
Electrophoresis device	ATTO CORPORATION	WSE-1150
$FeSO_4 \cdot 7H_2O$	FUJIFILM Wako Pure Chemical Corporation	098-01085
Glass filter 17G3	Tokyo Garasu Kikai Co., Ltd.	0000094147
Glycerol	FUJIFILM Wako Pure Chemical Corporation	070-04941
Hemocytometer	Funakoshi Co., Ltd.	521-10
High speed refrigerated centrifuge	KUBOTA CORPORATION	7780
Incubator	TAITEC CORPORATION.	G･BR-200
Incubator	TAITEC CORPORATION.	BR-43FL
KCl	FUJIFILM Wako Pure Chemical Corporation	163-03545
KH_2PO_4	NACALAI TESQUE, INC.	28721-55
Lysing enzyme	Sigma-Aldrich	L1412-10G
$MgSO_4 \cdot 7H_2O$	FUJIFILM Wako Pure Chemical Corporation	131-00405
Micro refrigerated centrifuge	KUBOTA CORPORATION	3740

Microscope	Leica Microsystems	DMI6000 B
NaCl	FUJIFILM Wako Pure Chemical Corporation	190-13921
$NaH_2PO_4 . 2H_2O$	NACALAI TESQUE, INC.	31718-15
$NaNO_3$	FUJIFILM Wako Pure Chemical Corporation	195-02545
Parafilm M	Bemis Company, Inc	PM-996
PCR Purification Kit	QIAGEN K.K	28104
Petri dish	Sumitomo Bakelite Co., Ltd.	MS-11900
Polyethylene glycol	Sigma-Aldrich	P3640-500G
Polypeptone peptone	Becton, Dickinson and Company	211910
Protein ladders	Bio-Rad Laboratories, Inc.	161-0377
Sodium dodecyl sulfate	Bio-Rad Laboratories, Inc.	1610301
Sterile filter	Merck KGaA	SLGP033RB
Sucrose	NACALAI TESQUE, INC.	30404-45
Tween 20	Tokyo Chemical Industry Co., Ltd.	T0543
Uridine	Sigma-Aldrich	U3750-25G
Yatalase	Takara Bio Inc.	T017

Comments/Description

Used for precast gel in SDS-PAGE
Used for SDS-PAGE

Used for centrifugation of samples in 50-mL conical centrifuge tubes
Used for flask liquid culture and preparation of protoplasts
Used for microplate liquid culture and plate culture

Used for centrifugation of samples in 1.5-mL microcentrifuge tubes

Used as culture plate

Used as molecular weight marker in SDS-PAGE

Point-wise Response to the Editor and Reviewers

Editor

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
   Response:
   To ensure that the manuscript is free of spelling and grammar issues, we have got it checked by an English language editing company.

2. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
   Response:
   As suggested by you, we have reviewed the description for each step of the protocol and have provided further details.

3. 3.1: Please specify the pore size of the filter.
   Response:
   We have specified the pore size (30 µm) of the glass filter.

4. 3.2: Please describe how the washing is done. Are the cells still on the filter?
   Response:
   We have added this information in the revised manuscript. The cells were on the glass filter during the washing process.

5. 4.1: Please specify the source of the DNA sample.
   Response:
   The DNA sample was a PCR-amplified fragment obtained using the plasmid as a template. This information has been included in the revised manuscript.

6. 5.1.4: Please explain what the target substances are and how to evaluate.
   Response:
   We have mentioned that the target substance was a heterologous protein, which was analyzed by SDS-PAGE.

7. Lines 173-207: Please include details of the methodology in the protocol section. The results section should focus on explaining how these results show the technique.
   Response:
   The description of the experimental method in the Representative Results section has been moved to the Protocol section, as suggested.

Reviewer #1:

Major Concerns:

Compared with the traditional PEG mediated transformation, the modified one in this study skipped the selection and screening steps for positive transformants in agar plates. However, the authors ignored that the modified method may make it difficult to obtain homozygotes, especially using the 200-ml flasks, because most transformants would not be stable in genetics or phenotype in a mixture culture. Besides, PEG mediated transformation method is widely applied in characterizing the function of unknown genes, detection the foreign gene product described in this manuscript was not the only purpose, and this simplified method using a nutrient-rich medium with optimized osmotic pressure lack more efficient selectable markers. Based on the above considerations, the application of the modified method would be limited.

Response*:*

We agree that the DLC screening method as described in this protocol is limited to secretory production of heterologous proteins using uridine auxotrophic mutants in the filamentous fungus, *Aspergillus oryzae*. Thus, we have changed the title to 'Direct liquid-culture screening method for evaluating the production of heterologous proteins using an auxotrophic mutant of *Aspergillus oryzae*.'

We acknowledge your comment regarding the limitations with respect to the application of the method and the use of selectable markers. As such, we have only claimed applicability of the DLC method for secretory production of heterologous proteins that does not require the acquisition of homozygotes. Regarding the limitation in the use of selectable markers, we have clearly mentioned in Representative results that, in its present form, the method is applicable when used with uridine auxotrophic mutant of *A. oryzae*. We have discussed this issue in the Discussion section of the revised manuscript (page 8, lines 332–337).

Minor Concerns:

1. Line 174, the accession number of CBH?

Response:

We have included the GenBank Accession Number (E39854) for CBH (page 6, lines 226).

2. In whole text, please make sure CBH or CBH1?

Response:

For consistency, we have used "CBH" throughout the revised manuscript.

3. Line 196, what is the relationship between osmotic pressure (use sorbitol in direct liquid-culture) and positive transformants?

Response:

In the DLC method, the transformation efficiency is basically considered as the combination of two factors: the viability of protoplasts and the gene transfer efficiency. The results in Fig. 3A represent the overall transformation efficiency. The rate of positive transformants can be assessed from the gene transfer efficiency, while in our experiments, we did not obtain the data. Yet we speculate that the effect of osmotic pressure on the gene transfer efficiency is relatively small compared to that on the viability of protoplasts and that PEG used in DNA introduction is primarily related to the gene transfer efficiency as reported by *Brzobohatý et al*. (1985) for *Saccharomyces cerevisiae*.

4. Line 224 and 233, how many days of culture supernatant used in SDS-PAGE?

Response:

We have included this information in the legends to Figure 3 (page 7, lines 284) and Figure 4 (page 7, lines 294).

5. Figure 4B, why did not show the PAGE of culture supernatant of the third transformant as the negative transformant?

Response:

We did not perform SDS-PAGE for the third transformant. We assessed that the gene had not been introduced into the third transformant because its growth was the same as of the sample in which the gene was not introduced (negative control). Even if the gene had been introduced into the third transformant, we presumed that no band would be observed after SDS-PAGE analysis because of the small amount of fungal biomass obtained. We have added the data for the negative control to Fig. 4A and 4B and the have provided information about the negative control in the Results section (page 6, lines 260–261) as well as in the legends to Fig. 4A and 4B.

Reviewer #2:

Major Concerns:

1. There should be some discussion on purification of strains. The DLC method does not purify the strains. How is this applicable to gene deletions or RNAi cassettes that don't have a phenotype that can be screened by SDS PAGE? If not, the title should reflect that it is only applicable to screen for secreted protein overexpression. Their abstract seems to state that it is for overexpression of proteins or secondary metabolites. I don't think they can claim it is for secondary metabolites without showing some data on the production of secondary metabolites.

Response:

Regarding the use of the DLC method, we only confirmed the secretion of heterologous proteins. Also, because confirmation by SDS-PAGE requires a distinct change in the secretion of the protein, we acknowledge that the application of the DLC screening method is limited to secretory production of heterologous proteins using uridine auxotrophic mutant of *Aspergillus oryzae*. We have, therefore, changed the title to 'Direct liquid-culture screening method for evaluating the production of heterologous proteins using an auxotrophic mutant of *Aspergillus oryzae.*'

2. Lien 269: Here it is claimed that the method is applicable to other gene modifications but presumably, these types would require strain purification?

Response:

We agree with the reviewer's contention and acknowledge the limitations of the described method. As such, in the revised manuscript, we have only claimed that the DLC screening method is applicable to secretory production of heterologous proteins using uridine auxotrophic mutants of the filamentous fungus, *Aspergillus oryzae*. For this application, it is possible to screen the target heterologous protein without purifying the strain.

3. The authors present a generic method for filamentous fungi but only show the results using one fungus. Have they tested other fungi?

Response:

We did not test filamentous fungi other than *A. oryzae*. For this reason, instead of making a generic statement regarding the use of filamentous fungi, we have specified that the DLC screening method is applicable for *A. oryzae*. For using other filamentous fungi in the DLC method, it would be necessary to optimize the conditions for protoplast formation and determine the ideal composition of the liquid medium. We have added relevant text in this regard to the Discussion section (page 8, lines 327–330).

4. Does this method work using antibiotic selection instead of using auxotrophic mutants?

Response:

Although we did not consider the use of antibiotic selection in the present study, a transformation method using antibiotics (e.g. bleomycin and pyrithiamine) instead of auxotrophic *A. oryzae* mutants has previously been reported. It may be possible to optimize the composition of the medium according to the antibiotic used. We have discussed this issue in the Discussion section of the revised manuscript (page 8, lines 332–337).

5. In Fig. 3, how is the non-transformed control culture grown? In Fig. 4, can the authors show the banding pattern from a non-transformed control grown in the 24 well plate. Also, the control and transformed culture samples should be run and stained on the same gel? Are these lanes from the same gel in Fig. 3?

Response:

In the experiment for which the results are shown in Fig. 3B, the negative control sample was a PD culture of spores of the WT strain. The data were obtained from an experiment different from the DLC screening. To clarify this, a dot blot has been inserted between each SDS-PAGE data in Fig. 3B. Regarding Fig. 4, the results of growth and SDS-PAGE of non-transformed sample have been added. Relevant descriptions have been added to the Results section (page 6, lines 260–261) and to the legends of Fig. 4A and 4B.

6. The authors need to show by another method that the Talaromyces cellulolyticus cellobiohydrolase (CBH) is produced such as shotgun proteomic analysis of the band excised from SDS-PAGE gel.

Response:

Because for production of CBH, we used the same expression cassette as previously reported by us (Mitsuzawa *et al*., 2017) and the size of the protein secreted by the transformed cells was also the same, we deduced that the band visualized on the gel is indeed of CBH. We have added the relevant description in this regard in the Results section (page 6, lines 254–255 and page 7, lines 266-267).

7. Are the authors exaggerating how long the "Conventional screening" takes at 21 days. For example the selection between true and false positive transformants could be skipped as the liquid cultures in the next step would also be able to do this. After acquiring the colonies in the first step, would it not be quicker to transfer these colonies to the 24-well plate (yes, it would involve more work to do this)?

Response:

It may be possible to skip the selection step by adding the colonies obtained in the first step of the conventional method directly to a 24-well plate. If possible, the DLC method would reduce the duration by four days relative to the conventional method. However, even in that case, the DLC method would reduce the man-hours. Usually, a plate culture is required for the acquisition and selection of colonies, but not in the DLC method. More is the number of samples handled, greater is the reduction in the man-hour using the DLC method. We have added new text in the Introduction section highlighting these points (page 2, lines 77–82).

Minor Concerns:

Why do you add cellulase to your lysing enzymes? It is a fungus not an oomycete that you are protoplasting.

Response*:*

We generated the protoplasts using the composition of enzyme solution described for *A.oryzae* strain RIB40 by *Tamano et al*. (2007). For the HO4 strain used in this study, it has been confirmed that protoplasts can be produced using only 1% Yatalase. Relevant descriptions have been added to the Discussion section (page 8, lines 327–330).

Reference:

Brzobohatý, B., Kováč, L. Interaction of plasmid DNA with yeast protoplasts and a mechanism of genetic transformation. FEBS letters. **183**(2), 211-214 (1985).

Mitsuzawa, S., Fukuura, M., Shinkawa, S., Kimura, K., Furuta, T. Alanine substitution in cellobiohydrolase provides new insights into substrate threading. Scientific Reports. **7** (1), 16320 (2017).

Tamano, K. et al. The β-1, 3-exoglucanase gene *exgA* (*exg1*) of *Aspergillus oryzae* is required to catabolize extracellular glucan, and is induced in growth on a solid surface. Biosci Biotechnol Biochem. **71**(4), 926-934 (2007).