For Biotechnology letters

Efficient and simple electro-transformation of intact cells for the basidiomycetous fungus *Pseudozyma hubeiensis*

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Abstract An electroporation procedure for the species was investigated to develop an efficient transformation method for the basidiomycetous fungus *Pseudozyma hubeiensis* SY62, a strong biosurfactant-producing host. A plasmid, pUXV1emgfp including green fluorescence protein as a reporter gene, was constructed to determine the transformation and expression of foreign genes. Optimal electroporation conditions achieved 44.8 transformants μg^{-1} plasmid competency (intact cells) without protoplast treatment. Lithium acetate treatments increased the efficiency to approximately twice that of control experiments. Almost all transformants demonstrated green fluorescence expressed in the transformant cells. The optimal method, successfully applied to several related species, yields sufficient transformant colonies to engineer the host strain.

Introduction

Pseudozyma hubeiensis is a basidiomycetous anamorph in the genus *Pseudozyma*, related to the teleomorphic fungus *Ustilago* (Wang et al. 2006). The *P. hubeiensis* species is isolated from leaves (Wang et al. 2006, and Konishi et al. 2007b) and deep sea invertebrates (Konishi et al. 2010). The species produces a glycolipid biosurfactant, mannosylerythritol lipid (MEL), from glucose and vegetable oils

(Konishi et al. 2007b and 2010). MELs are excellent surface-active compounds, and decrease surface tension in aqueous solution at considerably lower concentrations than required for chemical surfactants (Konishi et al. 2007b and 2010). The compound also show promising physicochemical properties and biochemical activity, including self-assembly (Imura et al. 2005 and 2007b), affinity for antibodies (Ito et al. 2007) and lectins (Konishi et al. 2007a), antimicrobial activity (Kitamoto et al. 1993), and cell differentiation activity (Isoda et al. 1997, Wakamatsu et al. 2001 and Zhao et al. 2001). MEL furthermore enhances gene transfection mediated by cationic liposomes by delivery of foreign genes into cells through plasma membrane fusion (Inoh et al. 2013). In cosmetic applications, MELs are capable of applying for hair and skin care products (Morita et al. 2010 and Yamamoto et al. 2012). The material possesses a great potential for a broad range of applications.

Pseudozyma hubeiensis SY62 is a significant host candidate for MEL production: its MEL productivity is the best of the reported MEL-producing strains (Konishi et al. 2011). The volumetric productivity reached 18.4 g MELs $l^{-1}d^{-1}$ in fed-batch cultivation (Konishi et al. 2011). The product consists of approximately 70% monoacetylated components, called MEL-C ((4-*O*-[4′-*O*-acetyl-2′,3′-di-*O*-alka(e)noil-β-D-mannopyranosyl]-D-erythritol)) (Fig. 1)(Konishi et al. 2010). Draft genome analysis of SY62 revealed that the gene cluster for MEL synthesis (*eml*1, *mac*1, *mac*2, *mmf*1, and *mat*1) is present on the genome. Compared to the other MEL producing strains, similarity of *mat*1 is low (Konishi et al. 2013). These sequence data provide also useful information for

55 the metabolic engineering using molecular biological technique. However, there is a major issue to be
56 addressed: the transformation procedure does not result in matured *Pseudozyma* cells.

57 There are few reports on the transformation of *Pseudozyma* and *Ustilago* cells with plasmid
58 vectors (Banks 1983, and Bej and Perlin 1989, and Kinal et al. 1991). Avis et al. (2005) reported that the
59 plasmid vectors pSceI-Hyg and derivatives were transferred into *Pseudozyma flocculosa* and *Pseudozyma*
60 *antarctica* by a method mediated by polyethylene glycol and calcium chloride with protoplast preparation.
61 They describe that three independent transformations in *P. flocculosa* gave only 5, 3, and 42
62 transformants for a foreign gene. Marchand et al. (2007) achieved good transformation efficiency of
63 100–200 transformants per μg of DNA per 10^8 cells by electroporation, and of 60–160 transformants per
64 μg DNA per 10^6 input cells by *Agrobacterium tumefaciens*-mediated transformation (ATMT), for *P.*
65 *antarctica*. Morita et al. (2007) described a convenient transformation of *P. antarctica* T-34 by
66 electroporation, with maximum transformation efficiency of 48 transformants per μg of plasmid DNA.
67 Using this method they also successfully obtained transformants of *Pseudozyma rugulosa* and
68 *Pseudozyma aphidis*. The efficiencies to these species were worse than those of well-known yeast such as
69 *Saccharomyces cerevisiae* (Manivasakam and Schiestl 1993 and Thomson et al. 1998) and *Pichia pastris*
70 (Wu and Letchworth, 2014). Protocols for *Ustilago* and for related *Pseudozyma* species reported have
71 never given transfomat of *P. hubeiensis* in our preliminary experiments. Therefore, transformation
72 condition should be optimized for each species.

We have investigated a simple and highly efficient transformation of *P. hubeiensis* intact cells by electroporation. The methods described above were not found suitable for *P. hubeiensis*, therefore we describe the optimization of the electroporation procedure in detail, including competent cell preparation.

Materials and Methods

Strains, growth media and culture conditions

Pseudozyma hubeiensis SY62 was provided by the Japan Agency of Marine and Earth Science and Technology. *Pseudozyma antarctica* NBRC 10260, *Ustilago maydis* NBRC 5346, and *Pseudozyma rugulosa* NBRC 10877 were purchased from the National Institute of Technology and Evaluation Biological Resource Center (NBRC). The strains were maintained as glycerol stocks at −80 °C. Liquid culture was performed in 200 ml baffled Erlenmeyer flasks containing 40 ml of yeast extract (3 g l^{-1}), malt extract (3 g l^{-1}), peptone (5 g l^{-1}) and glucose (10 g l^{-1}) (YM) broth for perpetrating glycerol stocks. Liquid cultures were directly inoculated with 100 µl of glycerol stock and incubated at 25 °C with 250 rpm orbital shaking. All transformants were grown on YM agar in the presence of the antibiotic hygromycin B (300 µg ml^{-1}).

Plasmid construction

Ustilago-Escherichia shuttle vector pUXV1 (ATCC 77463) was purchased from American

Type Culture Collection (ATCC). pPRSET-emGFP was obtained from Life Technologies. To construct pUXV1-emGFP, the GFP containing fragment emGFP fragment including green fluorescence protein was amplified by polymerase chain reaction (PCR) using the primers GFP-F-BamHI (AAAAAGGATCCATGGTGAGCAAGGG) and GFP-R-BamHI (AAAAAGGATCCTTACTTGTACAGCTCGTCCATGCC). The reaction mixture contained LA-taq polymerase (Takara Bio Inc. Shiga, Japan) 0.5 unit, dNTP mixture each 0.4 mM, pRSET-A-emGFP 0.1 μg, and 1.0 μM primers in 1×Takara LA-taq buffer. The thermal cycle reaction was performed at 94 °C for 5 min, 98 °C for 10 s to denature, 55 °C for 30 s to anneal, and at 72 °C for 1 min to elongate (28 cycles), then at 72 °C for 7 min in a T-100 thermal cycler (BioRad Laboratories, Inc., CA, USA). Amplified DNA fragments were purified using Wizard SV Gel and PCR Clean-Up System (Promega Co., WI, USA). The emGFP fragment and pUXV1 plasmid were cut with *Bam*HI and cloned into a *Bam*HI site on pUXV1 under the control of the *U. maydis gap* promoter, generating pUXV1-emGFP. The plasmid was sequenced with GFP-F-BamH1 and GFP-R-BamHI primers. A BigDye Terminator V3.1 cycle sequencing kit (Life Technologies) and ABI model 3130 capillary sequencer were used for sequencing. NucleoBond Xtra Midi Plus plasmid purification system (Takara Bio) was used up-scaled plasmid purification. The plasmid was digested by *Sac*I to linearize for the transformation experiments.

Transformation protocol

For the preparation of electrocompetent cells, the SY62 glycerol stock was directly inoculated into two independent fractions of 40 ml YPD medium (20 g l^{-1} pepton, 10 g l^{-1} yeast extract, 20 g l^{-1} glucose) in 200 ml baffled Erlenmeyer flasks. The cultures were incubated at 25 °C with 200 rpm shaking until the cell density reached 0.5 A (absorbance) at 600 nm in logarithmic growth phase. The cells were collected by centrifugation at 2,000×*g* for 5 min at 4 °C. The following procedure was used as a standard protocol expect as noted. The cell pellet collected from 80 ml of 1.0 A_{600} ml^{-1} culture broth was resuspended in 80 ml ice-cold pure water. The cells collected by centrifugation were resuspended in 40 ml ice-cold pure water. The cells were collected by centrifugation again, and washed with 10 ml of 1 M sorbitol. The washed cells were suspended in 400 µl of 1 M sorbitol. The cell suspension (80 µl) and 5 µg of DNA were transferred into an ice cold 2-mm gap vial, and incubated for 5 min on ice. The electroporation pulse was applied at 1,850 V, 25 µF and 1,000Ω using a GenePulser Xcell system (BioRad). Transformants were immediately diluted in 1 ml of ice-cold YPD broth, and incubated at 25°C for 3 h. Cells were collected by centrifugation and resuspended in 100 µl YPD broth. The aliquots were spread on YM agar containing 150 µg ml^{-1} of hygromycin. Transformed yeast colonies appeared after 5 days at 25°C. Competencies were calculated as the number of grown colonies per microgram of plasmid DNA.

Lithium acetate and dithiothreitol treatment

LiAc and DTT pretreatment were performed by Wu's method with slight modifications (Wu and Letchworth, 2014). Cultured and collected cells were resuspended in 100 mM Tris-HCl (pH 7.5) buffer with 100 mM LiAc and / or 10 mM DTT. The mixture was incubated at 25 °C for 30 min. In the control, the cells were resuspended in Tris-HCl buffer without DTT and LiAc, and incubated under the corresponding conditions. After pretreatment, cell preparation was performed using the standard procedure.

Microscopy

To examine the expression of GFP, one loop of each colony that appeared on the agar plate after transformation was transferred to 20 µl of autoclaved distilled water. After the sample was mixed, approximately 10 µl of sample was used for microscopy. Microscopy was performed using a Nikon ECLIPSE E800 microscope system (Tokyo, Japan) equipped with a Pixera 600CL-CV cooled color CCD camera (Pixera Japan, Tokyo, Japan). GFP fluorescence (10 s exposure time) was detected through a B-2A filter block (Nikon).

Results and discussion

Optimization of electroporation conditions

To enhance the transformation efficiency of *P. hubeiensis* SY62, the electroporation conditions

were optimized. *Saccharomyces cerevisiae* electroporation has previously been performed at 25 μF capacitance and 900V voltage (Delome 1989), and also at 1.5 kV, 25 μF, and 200Ω (Manivasakam et al. 1993). For *Pichia pastoris*, the electroporation pulse was reportedly applied at 1.5 kV, 25 μF and 186 Ω (Wu and Letchworth 2004). Under these conditions, a few hygromycin-tolerant colonies, 1.3 ± 0.9 colonies plate^{-1}, were observed. GFP-positive clones (showing green fluorescence under microscopy) were not detected. The above conditions seemed unsuitable for the transformation of *P. hubeiensis*.

A summary of our optimization results and electroporation conditions is in Table 1. An increase in resistance resulted in an increase in the number of hygromycin-tolerant colonies. With 800 Ω resistance (1500 V), competency reached 15 ± 8.0 colonies plate^{-1} and 3.0 ± 1.6 colonies μg^{-1}. At 1,000 Ω (1500 V), competency was 12 ± 5.5 colonies per plate and 2.4 ± 1.1 colonies μg^{-1} of competency, similar to those at 800 Ω. All detected colonies exhibited green fluorescence under microscopic analysis. As shown in Fig. 2, green fluorescence was observed from the transformants, but not from the wild type cells. These results suggested that a longer time constant (msec), calculated as the product of condenser capacity (μF) and resistance (Ω), may enhance the transformation efficiency. Therefore, we examined the effects of voltage (using 1,000 Ω resistance) on the transformation. The number of colonies at 1,150V was slightly lower than at 1,500V. At 1,850 V there were 224 ± 94 colonies plate^{-1}, and the competency reached 45±18 colonies μg^{-1} plasmid DNA. A larger voltage (2200 V) and longer time constant using a 50 μF condenser decreased the transformation efficiency. Morita et al. (2007) reported that a square pulse enhanced the

transformation efficiency for filtered *Pseudozyma antarctica* competent cells prepared in YM broth with 10% glycerol. However, GFP-positive transformants of *P. hubeiensis* SY62 were not obtained by this method, applying a square wave to competent cells prepared by our method. The number of colonies was 19 ± 10.8 colonies plate^{-1}, or 10% of the best results using the attenuating wave. A significant point of optimal electroporation condition for *P. hubeiensis* is long time constant 20-25 msec, which was longer than those of normal transformation method: for example, time constant is often set at 5 msec for conventional yeast including *Saccharomyces cerevisiae* (Manivasakam and Schiestl 1993) and *Pichia pastris* (Wu and Lethcworth 2004).

Effects of vector DNA concentrations

To examine optimal DNA concentration, transformations using 0.1, 1.0, 2.5 and 5.0 μg of DNA were carried out. Table 2 summarizes the effect of DNA concentration on the transformation efficiencies. At 5 μg of DNA, the colonies per plate and competency reached 129 ± 69 colonies plate^{-1} and 26 ± 14 colonies μg^{-1}, slightly less than the best of the above corresponding experiments (Table 1). These results indicate that each cell preparation causes different results, and implies that unknown factors in competent cell preparation affected the transformation efficiencies. The number of colonies per plate was lowered by a decrease in DNA concentration. Competency at 1.0 μg DNA was one order of magnitude smaller than that at 5 μg DNA. In case of 0.1 μg DNA, the competency was 50 ± 23 colonies μg^{-1}, however, the

181 number of colonies per plate was only 5.0 ± 2.3 colonies plate^{-1}. Considering the working efficiency for
182 transformation, this is not the best of the demonstrated conditions, and includes additional experimental
183 uncertainty due to the small numbers of transformant colonies. To minimize experimental uncertainty, the
184 optimization was carried out on the basis of numbers of colonies per agar plate. GFP fluorescence was
185 observed from over 90% of the transformants.
186
187 Effects of lithium acetate and dithiothreitol pretreatment
188 To further improve the efficiency, the effects of lithium acetate (LiAc) and dithiothreitol (DTT)
189 pretreatments on transformation were examined. In electroporation, DTT and LiAc pretreatment enhance
190 the transformation efficiency in *Saccharomyces cerevisiae* hosts (Thomson et al. 1998) and *Pichia*
191 *pastoris* (Wu and Letchworth, 2014). Table 3 indicates the effects of LiAc and DTT pretreatments on
192 transformation efficiency. In the control experiment without LiAc and DTT, the number of colonies and
193 competency were 107 ± 40 colonies plate^{-1} and 21 ± 8.2 colonies μg^{-1}. Although LiAc increased the
194 numbers of colonies and competency to 217 ± 57 colonies plate^{-1} and 43 ± 11 colonies μg^{-1}, respectively,
195 conditions including DTT reduced the efficiency by approximately 30% compared to the control
196 experiment. In *Pseudozyma antarctica*, a species taxonomically related to *P. hubeiensis*, it has been
197 reported that LiAc and DTT did not stimulate transformation efficiency (Morita et al. 2007). Therefore,
198 this difference of LiAc effect on transformation efficiency between *P. hubeiensis* and *P. antarctica* may be

dependent on the structure of cell surfaces and / or on experimental procedures not noted. The best efficiency of *Pseudozyma* was four orders of magnitude smaller than those of *Pichia pastris* (Wu et al. 2003). The low efficiency might be caused from the difference of cellular physiology, because the species show large phylogenetic distance between ascomycete and basidiomycete.

To examine the range of species for which this method is applicable, several species were transformed using the optimized conditions. Transformation efficiencies of *U. maydis* NBRC 5346, *P. antarctica* NBRC 10260, and *P. rugulosa* NBRC 10877 were 2.6 ± 0.1, 4.7 ± 0.9 and 29.6 ± 4.9 colonies μg^{-1}, respectively. Therefore, the optimized method could be applied to a broad range of species in the genera *Pseudozyma* and *Ustilago*. However, the efficiencies of related species were worse compared to that of *P. hubeiensis*. This implied that the optimal condition is different from those of related species.

In this study, the best transformation efficiency for *P. hubeiensis* resulted in approximately 200 colonies $plate^{-1}$ (as numbers of colonies) and 40 colonies μg^{-1} (as competency), when electroporation was carried out at 25 μF, 800–1,000 Ω, and 1,850 V using 5–10 μg DNA with 100 mM LiAc pretreatment. Although the efficiency in this study was slightly worse compared with the electroporation results of 100-200 colonies μg^{-1} DNA in *P. antarctica*, from a previous report (Marchand et al. 2007), our lower efficiency is probably caused by differences in the host and vector. Therefore, we consider that the efficiency using the current optimized conditions reached a satisfactory level for practical transformation.

We have described the first efficient transformation of *P. hubeiensis*, to the best of our

knowledge. Because *P. hubeiensis* SY62 is one of the best biosurfactant-producing strains, the transformation procedure, as a core technology for metabolic engineering techniques, will contribute to further development of biosurfactant production. Moreover, these results also provide useful information for optimization of transformation by electroporation in the taxonomically related species of the genera *Pseudozyma* and *Ustilago*.

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296

297 Figure legends

298 Fig. 1 Molecular structures of MEL. MEL-A: $R^1 = R^2 = CH_3C{=}O$-; MEL-B: $R^1 = CH_3C{=}O$-, $R^2 = H$;

299 MEL-C: $R^1 = H$, $R^2 = CH_3C{=}O$- (n = 2 - 14).

300

301 Fig. 2 GFP detection by microscopy. DIC indicates differential interface contact microscopy. FL indicates

302 fluorescence microscopy. Yellow scale bars are 10 μm.

303

Table 1. Effect of electroporation conditions for transformation

Resistance (Ω) (Ω)	Voltage (V) (V)	Number of colonies (colonies plate^{-1})	Competency (colonies μg^{-1})	GFP positive/negative (clones / clones)
200	1,500	1.3 ± 0.9	0.27 ± 0.18	0/1
400	1,500	1.3 ± 0.9	0.27 ± 0.18	2/2
800	1,500	15 ± 8.0	3.0 ± 1.6	25/1
1,000	1,500	12 ± 5.5	2.4 ± 1.1	23/0
1,000	1,150	4.6 ± 2.7	0.93 ± 0.54	3/4
1,000	1,850	224 ± 94	45 ± 18	30/0
1,000	2,200	66 ± 50	13 ± 10	18/0
1,000*	2,200*	n.d.	n.d.	-/-
Square wave**		19 ± 10.8	3.7 ± 2.16	0/11

* Condenser volume set at 50 μF

** Square wave input at 1,000 V for 1.0 msec twice with 5.0 msec interval.

The errors are standard errors, calculated from three individual experiments (n =3).

n.d. indicates not detected

Table 2. Effect of vector concentrations on transformation efficiencies

Vector (μg)	Number of colonies (colonies plate^{-1})	Competency (colonies μg^{-1})	GFP positive/negative (clones / clones)
0.1	5.0 ± 2.3	50 ± 23	8/0
1.0	5.3 ± 3.3	5.3 ± 3.3	8/0
2.5	94 ± 43	37 ± 17	27/3
5.0	129 ± 69	26 ± 14	27/3

The experimental errors indicate standard errors, calculated from three individual experiments (n =3).

Resistance and voltage were set at 1,000Ω and 1,850V, respectively.

Table 3. Effects of lithium acetate and dithiothreitol pretreatment on transformation efficiencies

Experimental conditions		Results		
LiAc (mM)	DTT (mM)	Number of colonies (colonies plate^{-1})	Competency (colonies μg^{-1})	GFP positive/negative (clones /clones)
0	0	107 ± 40	21 ± 8.2	30/0
100	0	217 ± 57	43 ± 11	30/0
0	10	68 ± 8.8	14 ± 1.8	30/0
100	10	68 ± 7.6	14 ± 1.5	30/0

The experimental errors indicate standard errors, calculated from three individual experiments (n =3).

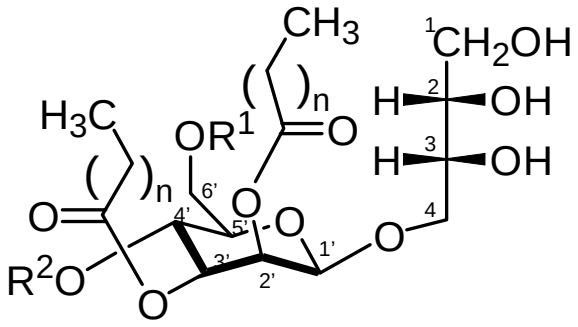

Figure 1. Konishi M. *et al.*

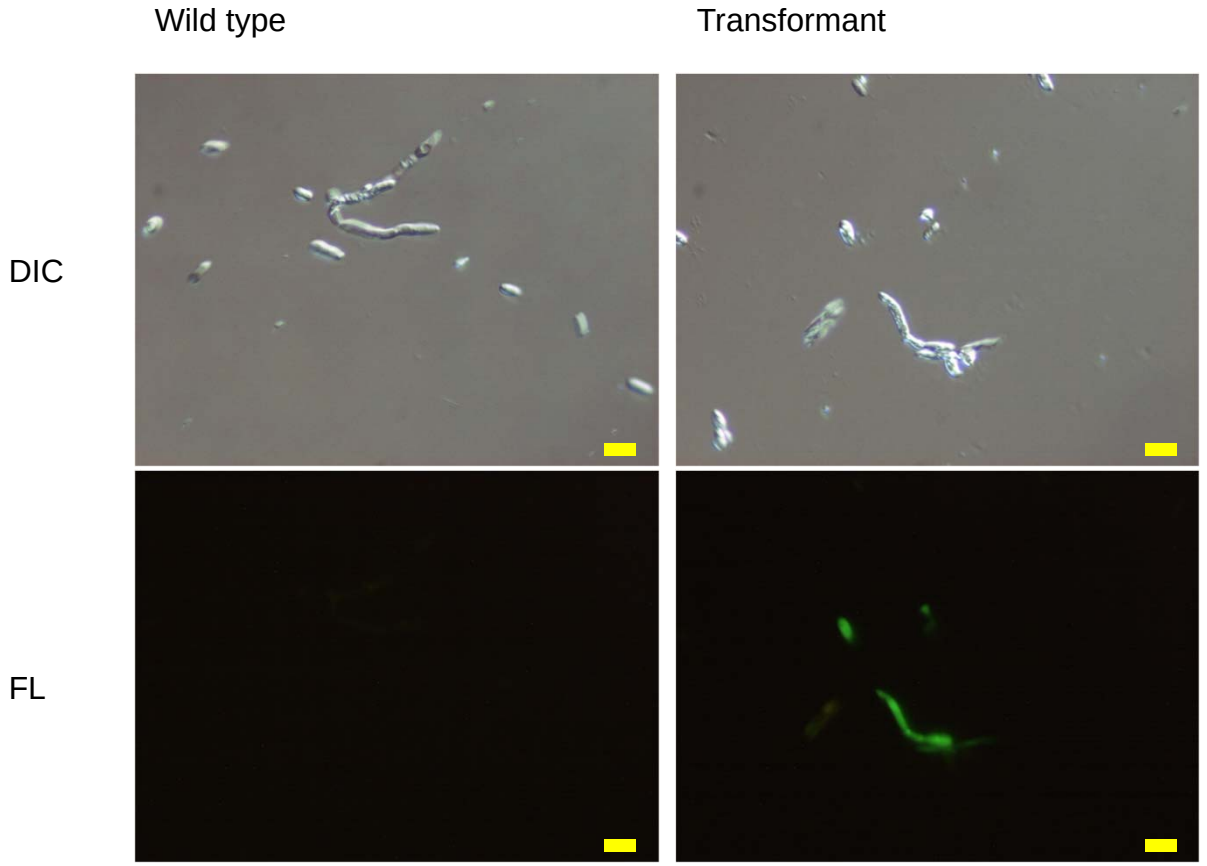

Figure 2. Konishi M. *et al.*