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High Efficiency Transformation by Electroporation of *Yarrowia lipolytica*

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***Yarrowia lipolytica* was usually transformed by heat shock, but linearized integrative vectors always resulted in a low transformation efficiency when electroporation was used. To develop a high efficiency integrative transformation method by electroporation of *Y. lipolytica*, we report here that pretreatment of *Y. lipolytica* with 150 mM LiAc for 1 h before electroporation will approximately 30-fold of increase transformation efficiency. A cell concentration of 10^{10}/ml and instrument settings of 1.5 kV will generate the highest transformation efficiencies. We have developed a procedure to transform *Y. lipolytica* that will be able to yield an efficiency of 2.1×10^4 transformants/μg for integrative linear DNA. With our modifications, the electroporation procedures became a very efficient and reliable tool for *Y. lipolytica* transformation.**

Keywords: dithiothreitol, electroporation, lithium acetate, transformation, *Yarrowia lipolytica*

Yarrowia lipolytica has been used in the manufacture of a variety of products for the past 60 years. Compared to other yeasts, *Y. lipolytica* has several useful properties, such as having the ability to metabolize several important industrial and agro-industrial by-products and to produce large amounts of Single Cell Oil (SCO) and organic acids (Finogenova *et al*., 2005; Rymowicz *et al*., 2007; Rywin'ska *et al*., 2009). Bankar *et al*. (2009) has presented the potential use and actual applications of wild-type, mutant, and recombinant strains of *Y. lipolytica* for different environmental and industrial applications. In addition, due to its dimorphic properties and its ability to widely express foreign proteins (Barth and Gaillardin, 1997; Cervantes-Chavez *et al*., 2009), *Y. lipolytica* is registered as a model microorganism.

Y. lipolytica is usually transformed by heat shock, but linearized integrative vectors like plasmid pYLEX1 always results in a low transformation efficiency (<1×10^4) (Chen *et al*., 1997). Chemical pretreatment of cells enhances transformation efficiency in other yeast (Thompson *et al*., 1998; Gietz and Woods, 2001; Suga and Hatakeyama, 2001). High efficiency transformation of *Schizosaccharomyces pombe* by electroporation requires pretreatment with dithiothreitol (DTT) (Suga and Hatakeyama, 2001). The transformation efficiency of *Saccharomyces cerevisiae* and its mutants are enhanced greatly by electroporation after combined lithium acetate (LiAc) and DTT pretreatment (Thompson *et al*., 1998). Wu and Letchworth (2004) found that *Pichia* transformation efficiency also can be enhanced approximately 150-fold when the cells are treated with LiAc and DTT prior to electroporation. But, Chen *et al*. (2008) reported that a combination of DTT and LiAc at the optimal concentrations for *Candida glycerinogenes* reduces transformation efficiency.

In the present work, the transformation efficiency of *Y. lipolytica*, treated with various combinations of LiAc and DTT before electroporation was examined. According to literature information, pretreatment with these chemicals has never been studied before with *Y. lipolytica*. A tremendous improvement of transformation efficiency was observed in cells of both tested strains treated with both LiAc and DDT. The effect of cell density on transformation efficiency of pretreated cells is also studied. The production of large numbers of transformants will enable efficient selection of multicopy transformants and random mutants exhibit desirable characteristics.

Materials and Methods

Strains, plasmids, and cultivation conditions

The expression host *Y. lipolytica* Po1g and the expression vector pYLEX1 were purchased from Yeastern Biotech. (Taiwan) (Fig. 1). A leucine gene (*LEU2*) in PYLEX1 provides for selection of yeast containing an integrated expression cassette by allowing their growth on leucine-free minimal medium (Madzak *et al*., 2000). *Y. lipolytica* cultures were usually grown at 28°C on YPD agar (10 g/L glucose, 10 g/L yeast extract, 10 g/L bacto peptone, and 2% agar) or YPD broth. For transformant selection, YNB medium (10 g/L glucose, 6.7 g/L yeast nitrogen base w/o amino acids, 15 g/L agar) was used. *Escherichia coli* DH5α (TaKaRa Bio, Japan) used for the propagation of plasmids was cultured in LB medium (Sambrook and Russell, 2001).

DNA manipulations

General recombinant DNA techniques were performed as described in Sambrook and Russell (2001). The plasmid pYLEX1 was propagated in *E. coli* strain DH5α, prepared by the QIAprep Spin Miniprep kit (QIAGEN, Germany), linearized by *Sac*II to produce an expression cassette that can integrate into the *Y. lipolytica* genome by homologous recombination. The DNA content was measured by Thermo Scientific NanoDrop 1,000 spectroscopy (Thermo Scientific, USA) at 260 nm. Restriction enzymes for DNA cleavage were used according to manufacturer's instructions (Fermentas, Lithuania). Primers pHP-XPtF (5′-ATATACAAGAGCGTTTGCCAG-3′) and pHP- XPtR (5′-CCAC CTACAAGCCAGATTTTC-3′) were synthesized based on sequence of plasmid pYLEX1. PCR which was performed by using *Y. lipolytica*

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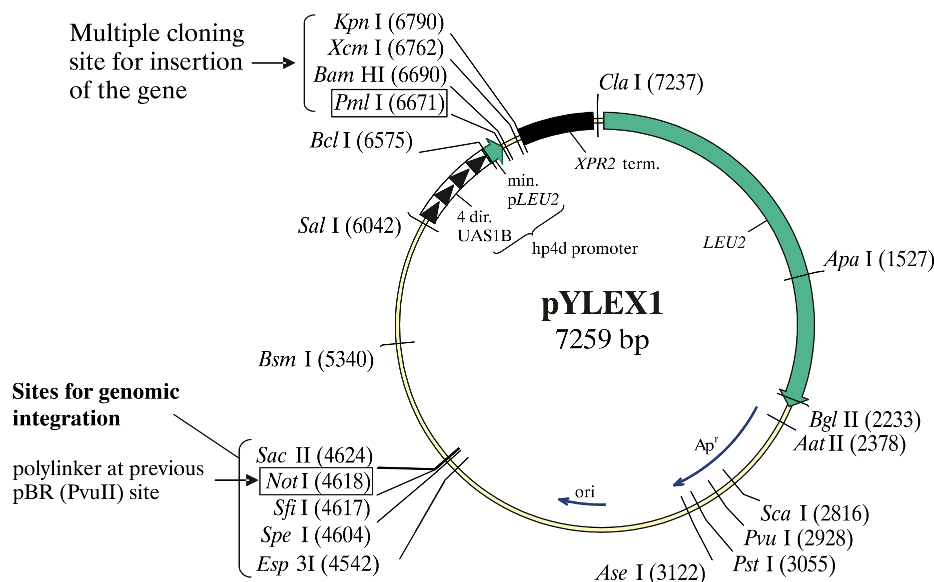

Fig. 1. pYLEX1 shows the position of genes and restriction sites. The pYLEX1 expression vector contains the strong hybrid promoter (*hp4d*) carrying four tandem copies of upstream activator sequence (*UAS1B*) fragment from p*XPR2* and a minimal p*LEU2* fragment. The multiple cloning sites and the *XPR2* transcription terminator lie immediately downstream of the 3′ site of the hp4d promoter, followed by a leucine selection marker gene (*LEU2*).

genomic DNA from the wild type and transformant strains as template with the primers pHP-XPtF and pHP-XPtR for 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and at 72°C for 60 sec.

Electroporation transformation procedure

The yeast was grown overnight in YPD broth with vigorous shaking (200 rpm). Twenty microliters of yeast culture were transferred to 100 ml of YPD broth and incubated at 28°C until the cell density reached 1-2 units of absorbance at 600 nm (A_{600}). Cells were pelleted by centrifugation, suspended at room temperature for 0 min, 15 min, 30 min, and 60 min with occasional shaking in 8 ml of 0.6 M sorbitol, with 10 mM Tris-HCl, pH 7.5 containing the indicated concentration DTT and/or LiAc. The cells were then pelleted, resuspended in 3 ml of ice-cold 1 M sorbitol, transferred to a 10 ml centrifuge tube, washed three times with 5 ml ice-cold 1 M sorbitol. This cell suspension was kept on ice until use. The cell suspension at a concentration of 10^{10} cells/ml (100 μl) was mixed gently with appropriate purified plasmid DNA. The number of cells was calculated by Nucleo Counter YC-100 (ChemoMetec, Denmark). After incubated on ice for 5 min, the mixture was immediately electroporated, using an electroporator with MicroPulser™ electroporation apparatus (Bio-Rad Labs, USA). Electroporation was performed by a single pulse at 1.5 kV (E=12.4 kV/cm), 200 Ω, and 25 μF (corresponding to pulse length of 4.6 ms), in 0.2 cm disposable electroporation cuvettes (Bio-Rad Labs). The electroporated cells were immediately diluted in 1 ml of ice-cold 1 M sorbitol, and 50 to 100 μl aliquots were spread on plates containing YNB medium.

Results

Effect of DTT or LiAc concentration on transformation efficiency

For transformation by electroporation, yeast cells were concentrated from a log-phase culture using multiple washes with sterile water to remove extracellular ions. The concentrated cells were suspended in 1 M sorbitol for osmotic stabilization. DNA is introduced without carrier, and the cells are transformed using an electric pulse delivered by an electroporation device. For maximum efficiency, yeast may be incubated with DTT and LiAc before concentration to render the cell wall more permeable. *Y. lipolytica* were treated with various concentrations of LiAc and DTT prior to electroporation. Optimal transformation efficiency was obtained when cells were pretreated with 150 mM LiAc (Table 1).

Table 1. Effect of DTT or LiAc concentration on transformation efficiency

Pretreatment condition	Concentration (mM)	Transformants/μg DNA
None		$6.77\pm0.42\times10^{2}$
DTT	10	$2.02\pm0.31\times10^{3}$
	25	$2.88\pm0.74\times10^{3}$
	50	$1.29\pm0.17\times10^{3}$
LiAc	50	$1.07\pm0.14\times10^{3}$
	100	$9.75\pm0.25\times10^{3}$
	150	$2.08\pm0.11\times10^{4}$
	200	$1.51\pm0.10\times10^{4}$
	300	$1.26\pm0.17\times10^{4}$
DTT+LiAc	10+100	$3.09\pm0.17\times10^{3}$
DTT+LiAc	25+150	$3.63\pm0.66\times10^{3}$

Cells of *Y. lipolytica* were pretreated with DTT or LiAc at room temperature with occasional shaking for 60 min. 30 ng linearized DNA was added to 100 μl cell and mixed. The results are the Mean±SD of three independent experiments.

Table 2. Effect of LiAc 150 mM pretreatment time on transformation efficiency

Pretreatment condition	Pretreatment time (min)	Transformants/μg DNA
None		$7.65\pm0.61\times10^2$
LiAc 150 mM	0	$8.33\pm1.57\times10^2$
	15	$1.04\pm0.17\times10^4$
	30	$1.12\pm0.13\times10^4$
	60	$1.93\pm0.24\times10^4$

Cells of *Y. lipolytica* were pretreated with DTT or LiAc at room temperature with occasional shaking for 60 min. 30 ng linearized DNA was added to 100 μl cell and mixed. The results are the Mean±SD of three independent experiments.

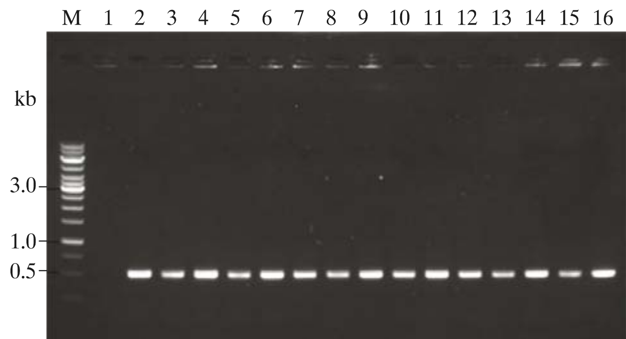

Fig. 2. Diagnostic PCR on transformants for confirmation of the integration of pYLEX1 into *Y. lipolytica* genome. Random transformants was identified by PCR with primers of pHP-XPtF and pHP-XPtR using colonies genomic DNA and *Y. lipolytica* genomic DNA as template. M, 1 kb ladder; Lanes: 1, *Y. lipolytica*; 2-16, transformants.

Effect of LiAc 150 mM pretreatment time on transformation efficiency

The transformation efficiency was increased with pretreatment time. After 1 h incubation, the transformation efficiency reached $1.93\pm0.24\times10^4$ transformants/μg DNA (Table 2). Our results demonstrated very clearly the effect of the 1 h incubation with LiAc before electroporation which increased transformation efficiency by approximately 30-fold. All of the transformants tested ($n=100$) showed normal growth, indicating that the number of false positive transformants was negligible.

Effect of cell concentration and voltage on transformation efficiency

The transformation efficiency varied directly with cell density (Table 3). At 10^{10} cells/ml, the transformation efficiency reached $2.17\pm0.21\times10^4$ transformants/μg DNA (Table 3). The transformation efficiency decreased with cell density. This result shows that high cell densities were critical to obtaining high transformation efficiency in *Y. lipolytica*. Voltage had the largest effect on transformation efficiency, 2 kV and 2.5 kV were significantly less efficient than 1.5 kV (Table 3). A very significant improvement in transformation efficiency was observed when cells were plated immediately after pulsing. The transformants were verified by PCR (Fig. 2). If the disruption cassette was integrated at the targeted locus, the 0.5 kb fragment corresponding from pHP-XPtF to pHP-XPtR should be amplified by PCR (Fig. 1). As indicated in Fig. 2, diagnostic PCR with primers pHP-XPtF and pHP-XPtR using genomic DNA of transformants as template showed that a 0.5 kb product was amplified, whereas no product was found using untransformed *Y. lipolytica* genomic DNA (Fig. 2).

Table 3. Effect of cell concentration and voltage on transformation efficiency

Cell concentration/ml	Voltage (kV)	Transformants/μg DNA
1×10^{10}	1.5	$2.17\pm0.21\times10^4$
	2	$1.16\pm0.15\times10^4$
	2.5	$8.73\pm0.89\times10^3$
5×10^9	1.5	$1.84\pm0.20\times10^4$
	2	$7.77\pm0.14\times10^3$
	2.5	$2.31\pm0.12\times10^3$
2.5×10^9	1.5	$1.70\pm0.27\times10^4$
	2	$6.06\pm1.35\times10^3$
	2.5	$2.72\pm0.16\times10^3$

Cells of *Y. lipolytica* were pretreated with DTT or LiAc at room temperature with occasional shaking for 60 min. 30 ng linearized DNA was added to 100 μl cell and mixed. The results are the Mean±SD of three independent experiments.

Discussion

In our study, the optimal LiAc concentration was higher than reported for other yeasts (De Backer *et al.*, 1999; Wu and Letchworth, 2004; Chen *et al.*, 2008). However, a combination of DTT and LiAc at the optimal concentrations reduced transformation efficiency. A similar phenomenon was also observed in the transformation of *Candida glycerinogenes* (Chen *et al.*, 2008). On the other hand, pretreatment of *S. cerevisiae* or *P. pastoris* with DTT/LiAc improved the transformation efficiency more than treatment with either component alone (Wu and Letchworth, 2004; Chen *et al.*, 2008).

We compared various known yeast transformation procedures. The incubation period with LiAc was set to 15 min, 30 min, or 1 h (De Backer *et al.*, 1999; Wu and Letchworth, 2004; Lin-Cereghino *et al.*, 2005; Chen *et al.*, 2008). Our results showed that treating *Y. lipolytica* cells with 150 mM LiAc during 1 h incubation is the optimal pretreatment time, while 15 min or 30 min incubation times are used for *S. cerevisiae* or *P. pastoris*.

Electroporation offers an excellent alternative to other, more time-consuming, transformation methods. Due to its ease, speed and efficiency in comparison to alternative techniques, with our modifications, the electroporation procedure becomes a very efficient and reliable tool for *Y. lipolytica* transformation.

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