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Optimization of *E. coli* culture conditions for efficient DNA uptake by electroporation

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Abstract: Bacterial transformation is a significant breakthrough in the field of molecular biology for cloning purposes. A variety of chemical and physical methods are utilized for enhancing bacterial competence. The present study was designed, via the consolidation of chemical and physical methods, to improve competence of cells for high transformation efficiency up to 10^9 transformants/µg of DNA. LB and SOC media and their supplemented combinations with osmotic agents (sucrose and glycine) were used to prepare electrocompetent cells of *E. coli* DH5α at temperatures of 25 and 37 °C, which were then subjected to electroporation. The results showed that cells grown in SOC medium showed high growth rates and increased transformation efficiencies as compared to LB, while media supplemented with osmotic agents also showed higher transformation proficiency as compared to the controls. In all combinations tested, the highest transformation efficiencies were observed in the cells grown in SOC-SG at 25 °C, i.e. 3.56×10^9 cfu/µg of DNA. This study provides valuable knowledge for low-budget biotechnology labs to prepare competent cells with efficiency comparable to commercially available competent cells.

Key words: Electroporation, optimization, culture conditions

1. Introduction

In molecular biology, transformation is the uptake or introduction and expression of foreign DNA by living cells (Roychoudhry et al., 2009). Many bacteria have the ability to uptake exogenous DNA from their close vicinity; this ability is termed as competence (Dreiseikelmann, 1994). There are 2 methods that are widely used for transformation: chemical and physical methods. In the chemical method, bivalent cations (Ca^{+2}, Mg^{+2}, Ba^{+2}, Rb^{+}, and Sr^{+2}) are chiefly used to induce competence in cells (Huang and Reusch, 1995). The use of bivalent cations to prepare competent cells increases their transformation efficiency. As reported by Beldüz et al. (1997), the use of CO_2-saturated calcium chloride at pH 4.6 increases the transformation efficiency of *E. coli* JM 1 09 cells by a factor of up to 6.3. Additionally, many other chemicals such as EDTA (Williams et al., 1996), polyethylene glycol (Himeno et al., 1984), and osmotic agents including sucrose (Antonov et al., 1993), glycine (Holo and Nes, 1989), sorbitol, and manitol (Xue et al., 1999) not only increase the chances of transformation but also improve the chances of cell survival when present in high concentrations in the electroporation medium.

Electroporation is the most common physical process of producing transient pores in the outer membrane of living cells. It works by subjecting them to a rapidly changing high-strength electric field. Electroporation is enhanced by several factors; Wu et al. (2010) reported that careful control of (i) cell growth phase, (ii) electroporation cell number, (iii) cell desalination with glycerol, and (iv) amount of transforming DNA may enhance transformation efficiency of *E. coli* up to 10^{10} transformants/µg DNA. The combination of chemical–chemical, chemical–physical, and physical–physical methods improves the transformation manifolds and is used in laboratories worldwide. Bukau et al. (1985) suggested that growth medium supplemented with sucrose facilitates DNA uptake. Transformation efficiency of *E. coli* may also be enhanced by the use of the simplest amino acid, glycine (1% w/v), in growth medium, as reported by Akhtar et al. (1999).

Although some basic methods (chemical and physical methods) are used for bacterial DNA transformation (Sheng et al., 1995; Neumann et al., 1996), they produce variable results under same laboratory conditions and in most cases results are not reproducible (Hengen, 1996). The objective of the present study was to develop an optimized protocol for high transformation yield of *E. coli* DH5α strain with reproducible results. To attain our

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objective, we studied growth rates and the transformation efficiency of *E. coli* DH5α in LB and SOC media in different combinations of osmotic agents (sucrose, sucrose+glycine) at different growth temperatures (25 and 37 °C).

2. Materials and methods

2.1. Bacterial strain and vector

The strain DH5α [*supE*44, Δ*lac*U169 (Φ80*lac*ZΔM15), *hsd*R17, *recA*1, *endA*1, *gyrA96*, *thi*-1, *relA*1] of *E. coli* was obtained from Plant Biotechnology Lab-1, UIBB PMAS-AAUR. The plasmid pENTR/D-TOPO gateway entry vector of size 2580 bp was used to produce recombinant vector pENTR/D-TOPO-RGLP1 of 3255 bp by ligation of the 675-bp-long *germin-like protein 1* gene (*RGLP1*) of rice (*Oryza sativa*). The vector harbors the kanamycin-resistance gene as a selection marker.

2.2. Media

LB (Luria Bertani: tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L), SOC (super optimal broth with catabolite: tryptone 20 g/L, yeast extract 5 g/L, NaCl 0.5 g/L, 250 mM KCl 1 mL/L), and their combinations with osmotic agents, labeled as LB (control), LB-S (LB medium supplemented with 40 mM sucrose), LB-G (LB medium with 1% glycine), LB-SG (LB medium supplemented with 40 mM sucrose and 1% glycine), SOC (control), SOC-S (SOC medium supplemented with 40 mM sucrose), SOC-G (SOC medium with 1% glycine), and SOC-SG (SOC medium supplemented with 40 mM sucrose and 1% glycine), were used for the preparation of competent cells. SOC medium was also used for postshock cell recovery. SOB medium with 50 mg kanamycin/mL was used for postshock plating of cells to find out cell survival and numbers of transformants/μg of DNA.

2.3. Growth kinetics

The growth kinetics of *E. coli* DH5α was studied in all the combinations of media given above at temperatures of 37 and 25 °C. A total of 2–3 mL of overnight incubated starter culture was inoculated in 100 mL of each medium in 2 sets of 250-mL flasks. One set was incubated at 37 °C while the second set was incubated at 25 °C, both with 250 rpm shaking. For monitoring the growth kinetics in each combination, optical density (OD) at a wavelength of 600 nm was taken after inoculation and then after each hour up to 12 h.

2.4. Preparation of electrocompetent cells

Electrocompetent cells of *E. coli* DH5α were prepared according to the standard protocol of Sambrook and Russell (2001) with certain modifications. In this study, competent cells were prepared in medium combinations of LB, LB-S, LB-SG, SOC, SOC-S, and SOC-SG at 2 different temperatures (25 and 37 °C), whereas in the standard protocol only the temperature of 37 °C and LB medium were used for cell harvesting. *E. coli* DH5α cultures were harvested at 0.4 OD_{600} by centrifugation at 3000 rpm for 10 min at 4 °C followed by 1 wash with ice-cold deionized water and 2 washes with ice-cold 10% glycerol prepared in deionized water. Pellets were then suspended in equal volumes of ice-cold 10% glycerol and 100-μL aliquots of *E. coli* DH5α were made, frozen in liquid nitrogen, and stored at –80 °C.

2.5. Electroporation

Competent cells were subjected to electric shock of 1.9 kV for 6 ms according to the following protocols.

2.5.1. Electroporation of LB and SOC control cells

Competent cells prepared in simple LB and SOC media were separated in aliquots of 50 μL in 1.5-mL Eppendorf tubes and mixed with 1 μL (1 μg/mL) of plasmid. The mixture was then transferred to a chilled electroporation cuvette (0.2 cm distance between electrodes). Voltage of 1.9 kV for 6 ms was applied. Immediately after shock, 1 mL of SOC medium was added, followed by shaking at 250 rpm at 37 °C for 1 h. The electroporated cells were then plated on kanamycin (50 mg/mL) SOB agar medium and grown overnight at 37 °C.

2.5.2 Electroporation of LB-S and SOC-S prepared cells

All steps were similar to the control, except that 270 mM sucrose was mixed with 50 μL of competent cells and 1 μL (1 μg/mL) plasmid just prior to electroporation.

2.5.3. Electroporation of LB-SG and SOC-SG prepared cells

All other steps of electroporation were similar to the control, except that 1% glycine and 270 mM sucrose were mixed with cells and plasmid.

2.6. PCR confirmation of plasmid

Plasmid was confirmed through PCR amplification after each transformation. The primers were constructed from GenBank based on the sequence of the plasmid. The sequence of M13 primers is given below:

M13 forward primers: 5′-GTAAAACGACGGCCAG-3′

M13 reverse primers: 5′-CAGGAAACAGCTATGAC-3′

Master Mix (10 μL) containing 3 μL of DNA template (50 ng/μL), 1 μL of Taq buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, and 0.8% Nonidet P40), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1.5 U of Taq DNA polymerase (recombinant; Fermentas, Lithuania), and 0.5 μL of each primer (100 ng/μL) were used to perform PCR. The thermal profile of PCR was initial denaturation of 95 °C for 3 min followed by 30 cycles of denaturing at 95 °C for 20 s, annealing at 58 °C for 20 s, and extension at 72 °C for 40 s, with a final extension at 72 °C for 10 min. Positive and negative control reactions were also set up. The PCR yields were then examined by

electrophoresis on 1% agarose gel prepared in TAE buffer (Tris-acetate 40 mM, 1 mM EDTA, pH 8.1) according to the protocol of Sambrook and Russell (2001).

2.7. Statistical analysis

The data were then analyzed by 2-way ANOVA, followed by least significant difference (LSD) test using MSTATC software.

3. Results

3.1. Assessing efficient growth of *E. coli* via different media and temperature combinations

The growth rate of *E. coli* DH5α was monitored at 37 and 25 °C in LB and SOC media and their supplemented combinations with 40 mM sucrose (LB-S and SOC-S), 1% glycine (LB-G and SOC-G), and 40 mM sucrose with 1% glycine (LB-SG and SOC-SG). In all combinations of LB tested at 37 °C, an enhanced growth rate was observed in the presence of sucrose, while the combination of sucrose and glycine showed only an intermediate effect on growth. A very low growth rate was observed in the presence of glycine as compared to all combinations tested, as shown in Figure 1. Glucose-containing nutrient-rich SOC medium was more efficient compared to LB for achieving the required OD_{600} of 0.35–0.4 for electrocompetent cell preparation, as observed in Figure 2. In all combinations tested, SOC in combination with sucrose proved to be a better osmotic agent for achieving rapid growth rate. Cells grown in SOC-S medium at 37 °C reached the midlog phase within 1 h after inoculation and SOC-SG took approximately 2 h (Figure 2), whereas LB-S and LB-SG took more than 2 h to achieve the required density for competent cell preparation (Figure 1). Conversely, at 25 °C all combinations of LB and SOC tested took more than 5 h to achieve midlog phase; however, SOC-S and SOC-SG reached midlog phase earlier than other combinations of osmotic agents tested (Figures 3 and 4). We deduced from our results that SOC-S and SOC-SG are better media combinations for achieving the required density at both temperatures in less time.

3.2. Assessing enhanced cell survival and transformation efficiency

The experiments investigated the effects of sucrose and sucrose+glycine on transformation efficiency when added to media and transformation mixture. Glycine supplementation was skipped in subsequent steps of transformation because of reduced growth rates. SOC and its supplemented combinations had pronounced effects on cell survival compared to LB. However, the highest cell survival was observed in cells grown in sucrose-supplemented SOC medium at 25 °C, as shown in Table 1. The increased survival rate was possibly due to the presence of extra nutrients in SOC medium and supplementation of sucrose as an osmoticum during growth as well as at the time of electrotransformation.

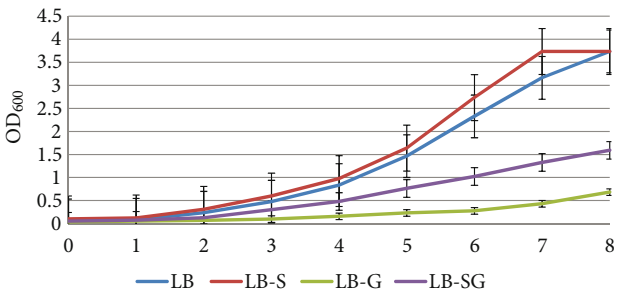

Figure 1. Growth rate of *E. coli* at 37 °C in LB with or without supplements.

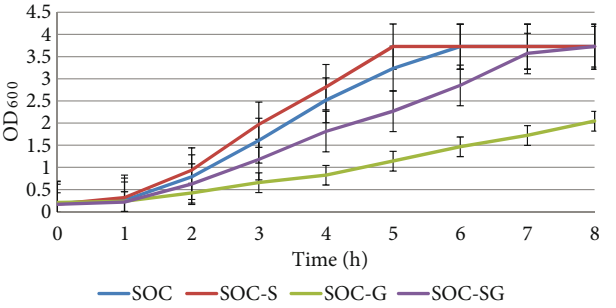

Figure 2. Growth rate of *E. coli* at 37 °C in SOC with or without supplements.

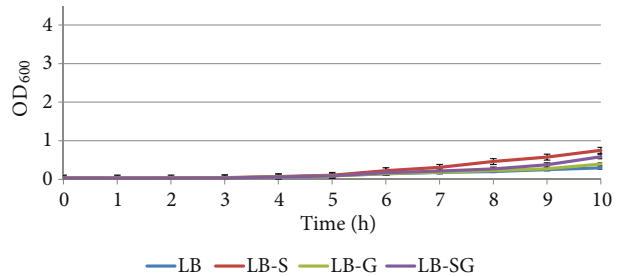

Figure 3. Growth rate of *E. coli* at 25 °C in LB with or without supplements.

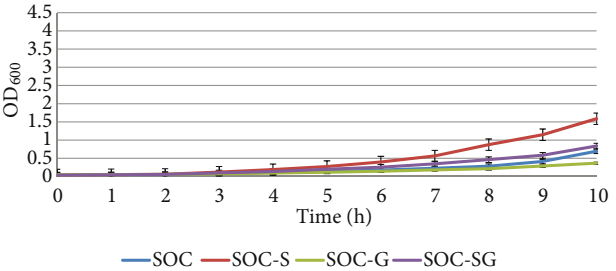

Figure 4. Growth rate of *E. coli* at 25 °C in SOC with or without supplements.

Table 1. Postshock % cell survival of *E. coli* grown in LB and SOC media supplemented with sucrose (40 mM) and sucrose+glycine (40 mM and 1%) at 25 and 37 °C and electroporated with supplementation of sucrose (270 mM) and glycine (1%) in transformation mixture. Least significance difference (LSD) for osmotic agents and media at 25 and 37 °C are shown with superscripted letters.

Replications	Temperature											
	25 °C						37 °C					
	LB	LB-S	LB-SG	SOC	SOC-S	SOC-SG	LB	LB-S	LB-SG	SOC	SOC-S	SOC-SG
1	27.6	30.5	27	44.6	51.2	47.8	24.7	22.4	33.3	38	45.7	38.1
2	30.9	30.8	34.7	42.6	50	45.4	23.2	18.1	27	38.4	40.7	38.3
3	25.2	34.5	23.8	45.5	52.4	53.7	30.4	23.1	33.3	41.6	37	36.6
Average ^{LSD}	27.9^{ef}	32^{cd}	28.5^{ef}	44.4^{ab}	51.2^{a}	49^{ab}	26.1^{ef}	21.3^{ef}	31.2^{de}	39.3^{bc}	41.1^{b}	37.7^{cd}
Change (fold)	Control	1.15	1.02	1.59	1.84	1.76	0.94	0.76	1.12	1.41	1.47	1.35

Two-way ANOVA was applied to the results presented in Table 1 to determine the significance of cell survival among osmotic agents in LB and SOC media at 37 and 25 °C. A highly significant ($P < 0.01$) difference of cell survival among osmotic agents in LB and SOC media was observed. The lower temperature (25 °C) had a better effect on survival, though the growth remained slower. Further analysis of results with LSD testing was carried out to determine differences among treatments. LB-S medium at 37 °C had the lowest percentage of cell survival, while LB (control) at 37 °C had somewhat better survival. The highest percentage of cell survival was observed for SOC-S and SOC-SG media at 25 °C as compared to other combinations (Table 1).

In the case of transformation efficiencies, we observed an increase in transformation efficiency in SOC media as compared to LB (Table 2). The highest transformation efficiency was observed in the case of SOC-SG at 25 °C (i.e. 3.56×10^9 transformants/µg of plasmid DNA) and this transformation efficiency was 49-fold as compared to the LB control. The results of LB and SOC media at 25 and 37 °C were statistically analyzed with the help of 2-way ANOVA. A highly significant ($P < 0.01$) transformation efficiency increase of *E. coli* DH5α was observed when cells grown in SOC and its supplemented combinations at 25 and 37 °C were subjected to electroporation. The difference in transformation efficiency at both temperatures was nonsignificant ($P > 0.05$). However, the results were further analyzed with LSD testing to determine the difference between osmotic agents used. The results indicated that all treatments of LB (LB control, LB-S, and LB-SG) at both temperatures (25 and 37 °C) had almost equal effect on transformation efficiency, which was less than that of SOC, while all combinations of SOC (SOC control, SOC-S, and SOC-SG) at both temperatures (25 and 37 °C) demonstrated variable results. The highest transformation efficiency was observed in the case of SOC-SG medium and 25 °C temperature. The success of

Table 2. Comparison of transformation efficiency of *E. coli* cultured in sucrose- and sucrose+glycine-supplemented LB and SOC media at temperatures of 25 and 37 °C. LSD results are depicted with superscripted letters; the highest transformation efficiency was observed in the case of SOC-SG at 25 °C. In addition, the relative increase (fold) was calculated for the data obtained.

Replications	Temperature											
	25 °C						37 °C					
	LB	LB-S	LB-SG	SOC	SOC-S	SOC-SG	LB	LB-S	LB-SG	SOC	SOC-S	SOC-SG
1	7.2×10^7	2.4×10^8	1.9×10^8	9.3×10^8	2.89×10^9	3.22×10^9	5.5×10^7	0.85×10^8	2.4×10^8	7.21×10^8	1.71×10^9	2.34×10^9
2	9.5×10^7	1.6×10^8	2.0×10^8	7.7×10^8	3.59×10^9	3.45×10^9	6.2×10^7	1.1×10^8	1.3×10^8	6.46×10^8	1.56×10^9	2.17×10^9
3	5.3×10^7	1.8×10^8	1.3×10^8	6.6×10^8	3.41×10^9	4.00×10^9	2.7×10^7	1.0×10^8	1.9×10^8	8.10×10^8	1.34×10^9	1.76×10^9
Average	7.3×10^7	1.9×10^8	1.7×10^8	7.9×10^8	3.30×10^9	3.56×10^9	4.8×10^7	1.0×10^8	1.9×10^8	7.26×10^8	1.54×10^9	2.10×10^9
Fold increase ^{LSD}	Control	2.6^{e}	2.3^{e}	10.8^{de}	45.2^{ab}	48.8^{a}	Control	2.1^{e}	4.0^{e}	15.1^{e}	32.1^{cd}	43.8^{bc}

each transformation experiment was validated after each transformation by isolating recombinant vector pENTR/D-TOPO-RGLP1 of 3255 bp and its amplification via M13 primers as shown in Figure 5.

4. Discussion

We presume from our results that SOC-S and SOC-SG are better media combinations for achieving the required density of 0.3–0.45 for preparing competent cells at both temperatures in less time, as evident from the comparison of results depicted in Figures 1–4. However, efficient growth kinetics of *E. coli* are not an indication that it will transform efficiently; Wu et al. (2010) reported that transformation efficiency is inversely proportional to cell population size. The increased growth rate in the presence of SOC medium might be due to the fact that SOC is a glucose-containing nutrient-rich medium, and glucose is a readily metabolizable sugar (Tu et al., 2005). However, the accelerated growth rate in SOC-S medium might be due to increased levels of sugars, as sucrose is metabolized into glucose and fructose. In contrast, glycine when added to growth media may interfere with the biosynthesis of membranes by replacing L- and D-alanine from peptidoglycan with glycine and thus reducing cell growth (Kaderbhai et al., 1997). As glycine is metabolized into acetate and acetate is an inhibitor of bacterial growth in this way, it reduces the growth rate of *E. coli* (Luli and Strohl, 1990). The protocol of Sambrook and Russell was followed in preparing electrocompetent cells, with modifications of media combinations and temperature. The modified protocol yielded promising results when SOC-S and SOC-SG were used for preparing competent cells at both temperatures.

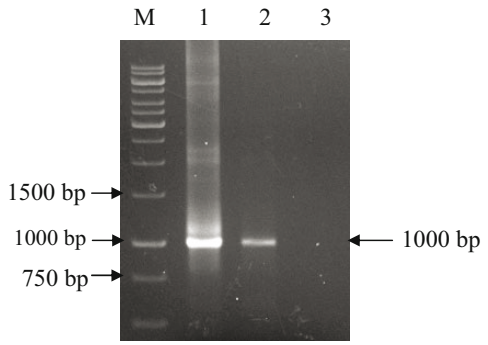

Figure 5. Agarose gel electrophoresis of PCR amplified *RGLP1* from RGLP1-pENTR/D TOPO vector DNA. Lane M: Gene Ruler 1 kb DNA ladder (Fermentas, Lithuania). Lane 1: PCR product of plasmid pENTR/D-TOPO: *Os*RGLP1, presence of 1000 bp fragment confirmed transformation of *E. coli* DH5α with pENTR/D-TOPO: *Os*RGLP1. Lane 2: PCR product of positive control (*Os*RGLP1). Lane 3: Negative control without any template.

Furthermore, the high transformation efficiencies observed in all combinations of SOC media might be due to presence of a bivalent cation, $MgCl_2$, which helps to facilitate DNA uptake. Likewise, supplementation of sucrose (270 mM) and glycine (1%) in the transformation mixture further enhanced transformation efficiencies as they acted as osmotica. Chehal et al. (2007) observed an increased transformation rate in *E. coli* grown in LB medium supplemented with 0.4 M sucrose. Wang et al. (2007) used 40 mM sucrose in *E. coli* growth medium and subjected the cells to electroporation followed by a heat pulse, resulting in a 250-fold increase in transformation efficiency. It was reported by Eynard et al. (1992) that transport of DNA into the cell was facilitated by influx of sucrose. Recently, Safia et al. (2014) reported that the use of osmotic agents (sucrose, glycine, and β-mercaptoethanol) during growth in LB medium and subsequent transformation mixture positively affected transformation efficiency. Our results show similar patterns of increased transformation and cell survival in the presence of sucrose, both in LB and SOC media, which might be due to the mass flow of sucrose into the cells when it was used in transformation mixture (Tables 1 and 2). However, the presence of glycine in the transformation mixture further enhances transformation efficiencies. As reported by Kaderbhai et al. (1997), glycine may impair *E. coli* membrane synthesis by replacing the L-alanine and D-alanine found in peptide units of peptidoglycans, hence making the membrane more permeable to macromolecules. In addition to the role of glycine in structural impairment, Kurkdjian et al. (1993) reported that glycine acts as an osmoticum and increases the permeability of the membrane. Hence, the addition of glycine to the transformation mixture helps the uptake of exogenous DNA present in the transformation mixture (Bukau et al., 1985). Thus, the increased transformation efficiencies observed in presence of sucrose and glycine, as shown in Table 2, might be due to structural impairments of the bacterial cell wall; furthermore, the osmotic agents might have facilitated DNA uptake.

In addition to media combinations of osmotic agents, we also tested temperature as a parameter for enhancing electroporation efficiency. Our results depict an enhanced electroporation rate at lower temperature (25 °C) as compared to the optimum temperature for DH5α growth, as demonstrated in Table 1. When cells are grown in temperatures lower than the optimum temperature of 37 °C, the proportion of unsaturated fatty acids in their membranes increases (Gill and Suisted, 1978), which increases fluidity and can interfere with membrane functions (Kuo et al., 1990), thus making the membrane more permeable to DNA.

In conclusion, this study supports previous findings that osmotic agents and lower temperature are imperative

factors in enhancing DNA uptake efficiency. However, in the present study it was concluded that an efficient culture medium for increasing the number of transformants/µg of DNA is SOC medium supplemented with sucrose and glycine, as compared to LB. This investigation reinforces the notion that even small/low-budget molecular biology laboratories could efficiently prepare highly efficient electrocompetent cells with transformation efficiencies of 3.56×10^9 cfu/µg DNA.

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