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In vivo expression of a short peptide designed from late embryogenesis abundant protein for enhancing abiotic stress tolerance in *Escherichia coli*

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Abstract

In vivo functional analyses of a late embryogenesis abundant (LEA) short peptide expressed in recombinant *Escherichia coli* BL 21 (DE3) were carried out under abiotic stress (salt, heat, and cold) conditions. Our LEA peptide was derived from the *Polypedilum vanderplanki* group 3 LEA protein based on distinctive conserved amino acid motif sequences. We focused on high-salt (5% and 7% NaCl) concentrations to evaluate the functional relevance of the peptide under abiotic salt stress. *E. coli* transformants expressing the LEA peptide showed higher cell viability than the control not expressing the peptide when transferred to a medium containing 5% and 7% NaCl; cells expressing LEA peptide showed a higher number of colony-forming units per dilution under the high salt stress condition. Moreover, expression of the LEA peptide resulted in greater cell survival under heat (48°C) and cold (4°C) stress. These results suggest that LEA short peptide co-expression could be useful for developing genetically modified organisms and in applications to prevent *E. coli* cell death under high salt, heat, and cold stress.

Keywords: *Escherichia coli*, LEA peptide, abiotic stress tolerance, late embryogenesis abundant protein

Abbreviations: IPTG, isopropylthio-D-galactoside; LB, Luria–Bertani medium; LEA, late embryogenesis abundant

Highlights

- A peptide of late embryogenesis abundant (LEA) protein was expressed in *E. coli*
- LEA peptide expression was induced with IPTG in a concentration-dependent manner
- LEA peptide expression improved *E. coli* growth under salt stress at 5% and 7% NaCl
- LEA peptide expression enhanced cell survival under heat and cold stress
- LEA peptide could be used for genetic modification in adaptation to abiotic stress

1. Introduction

Environmental accentuation such as increased soil salinity, water deficiency, and extreme temperature is the major limiting factor for the growth and productivity of all organisms. Several physiological and biochemical strategies have been developed to help organisms better adapt to or tolerate various abiotic and biotic stress conditions [1,2]. At present, there is great research effort focused on genetic manipulation strategies to enhance the accumulation of low-molecular-weight osmolytes that can help to increase tolerance to water or salt stress in genetically modified organisms [3]. A large set of protein-coding genes can be transcriptionally activated to develop a stress response [4]. However, there is still no detailed information on the influence of the expression of small biomolecules such as peptides *in vivo* for developing a suitable response against abiotic stresses.

The expression of the late embryogenesis abundant (LEA) gene and protein tends to be upregulated under stress conditions in many species. LEA protein was first identified from a cotton seed during late embryo development, and can be classified into several groups on the basis of common amino acid sequences. Group 3 LEA proteins have a distinct number of repetitions of the 11-mer motif or repeated amino acids configured as an amphipathic alpha-helix (TAQAAKEKAGE), which determines the molecular mass of this group [5, 6]. Several LEA proteins and dehydrins have been discovered in plants and animals to date, which act as molecular chaperones to protect the membrane proteins, cell organelles, activity of enzymes, and nucleic acids under various stress conditions [7]. Although the specific mechanisms of protection are unclear, some LEA proteins have been shown to function as ion scavengers, molecular chaperones, or shields of macromolecules to avoid protein aggregation and restore improperly folded proteins under dehydration, heat, or freeze-thaw stresses [7-10].

LEA protein expression confers tolerance in response to salt [3, 11,12], temperature [11, 13], drought [11, 14] and osmosis [14] as well as some signaling molecules, in many plant species, invertebrates, and higher and lower microorganisms. The aquatic larvae of *Polypedilum vanderplanki* survive under drought in an almost completely dehydrated state during the dry season in a semi-arid area. This phenomenon, termed "anhydrobiosis", has been attributed to expression of its LEA proteins [15].

In our previous study, we designed and constructed an LEA peptide co-expression system, which was used to target green fluorescent protein (GFP) and some other proteins for enhanced expression in *E. coli* BL21 (DE3) [16]. For production of the recombinant protein in *E. coli*, we designed LEA peptide sequences incorporated with the pRSF-Duet Dual vector, which were based on the 11-mer motif repetitive sequence of *P. vanderplanki* LEA (PvLEA) proteins [17]. Expression of the target protein (GFP) in *E. coli* was enhanced relative to that of the control strain that did not express the LEA peptide. We hypothesized that these LEA peptides function after translation to act as a molecular shield for stabilizing and protecting the target protein from lysozyme activity [16, 18].

The aim of the present study was to further explore the potential utility of LEA peptides as biological protectants during abiotic stress from a high salt concentration. The initial objective was to clone and overproduce a target protein using our previous LEA peptide co-expression system. Although the specific functions of LEA proteins remain unknown, they are generally assumed to play important roles in the establishment of environmental stress tolerance in many species, and could therefore serve as promising biological protectants under stressful abiotic and biotic conditions [7-15,17,19]. Therefore, the next objective of this study was to determine whether the synthetically designed peptide could play a vital role in protecting *E. coli* under a

high salt condition *in vitro*. The LEA peptide expression vector was constructed and transformed into *E. coli* BL21 (DE3), and its effects on bacterial cell resistance to stress was examined. These results can provide a new tool for enhancing stress tolerance in genetically modified organisms for various applications, and provide insight into the function of the LEA peptide 11-mer motif sequence and t he general mechanism of the response to abiotic stress.

2. Materials and methods

2.1. Peptide design and plasmid construction

The 13-mer peptide MDAKDGTKEKAGE was used as a model of LEA proteins originating from *P. vanderplanki* based on repeats of the 11-mer consensus motif, characteristic of Group 3 LEA proteins. The details on plasmid construction for peptide expression in *E.coli* BL21 (DE3) is provided in our previous paper [16].

2.2. Cell culture

E. coli BL21 (DE3) cells carrying the recombinant pRSF-LEA I vector were grown at 37°C in Luria–Bertani (LB) medium supplemented with 50 µg/ml kanamycin. For expression induction, different concentrations of isopropylthio-D-galactoside (IPTG), 0.0 mM, 0.01 mM, 0.1 mM, 0.5 mM, and 1.0 mM, were added to the cell cultures at an optical density at 600 nm (OD_{600}) of 0.5, and the cultures were grown for 24 h.

2.3. Tolerance and growth capacity of E. coli under salt stress

Cell cultures were grown as described above, and IPTG was added to mid-log phase cultures ($OD_{600} = 0.5$) at a final concentration of 0.0 mM, 0.01 mM, 0.1 mM 0.5 mM, and 1 mM, and

incubation was continued at 37 °C for 2 h. After IPTG induction, the cultures were incubated at 37°C (120 rpm). The bacterial suspensions (1 ml) were taken at 4 h, and diluted in ten-fold serial steps up to the 10−6 dilution stage. From each diluted suspension, 50 µl was spread on the LB agar plate; the OD_{600} values from each sample were checked to confirm equal concentrations. For salt treatment, after IPTG induction, 50 µl of each sample was spread onto the LB plates (while again controlling the OD_{600} for each sample) containing phosphate buffer or 3% NaCl, 5% NaCl, and 7% NaCl, respectively. After the plates were incubated for 1 to 2 days at 37 °C, the number of colony-forming units on each plate was recorded. The survival ratio was calculated using the following equation:

Survival ratio = (mean colony number on the salt plate/mean colony number on the control LB plate) × 100%.

2.4. Heat and cold shock tolerance

Evaluation of cold and heat tolerance was performed based on the growth of transformed *E. coli* BL21 (DE3) cells with pRSF-LEA I plasmids with different concentrations of IPTG. The cell cultures were incubated in LB liquid medium with IPTG for 4 h. For the thermophylactic experiments, the induced product was transferred to 1ml aliquots heated at 48 °C for 30 min, and then 100 µl of the serial dilutions were plated onto LB plus kanamycin plates. Cold shock tolerance was evaluated by exposure to cell cultures at 4 °C for 24 h. One milliliter of the induced cultures (OD_{600} = 0.9–1.0) was cooled at 4 °C for 24 h, and then 50 µl was transferred to the LB agar plate and cultivated at 37 °C. The cell growth experiment was repeated three times, with essentially the same results obtained.

2.5. Cell viability assay

Cell viability was measured using a colorimetric assay on 96-well plates with WST reagent and dimethyl sulfoxide (Microbial viability assay kit-WST, Dojindo, Kumamoto, Japan). Each plate contained blanks, controls, and 5% and 7% salt LB broth with stressed recombinant *E. coli* harboring the pRSF-Duet plasmid with or without 0.1 mM IPTG, with five replicates for each dilution series. Cells were added to the plates at 0.5 cells/ml and cultivated for 2 h. After 4 h, 20 µl of WST (diluted 1:4 with phosphate buffer) was added and 180 µl cells were incubated for an additional 4 h. The absorbance was measured on a microplate reader (Perkin Elmer, Waltham, MA) at 450 nm, with filters at ƛmax 460 nm, and the percent cytotoxicity was calculated as the percentage cell viability (relative to the WST-dimethyl sulfoxide reduction) compared to controls. The cell viability assays were generally carried out for a 1:1 dilution series in the concentration range.

3. Results

3.1. Effect of LEA peptide expression in E.coli on salt tolerance

E. coli cells expressing the short LEA peptide were exposed to 3%, 5%, and 7% NaCl as harsh conditions for survival and growth. No colony of *E. coli* BL21 (DE3) without the expression of LEA peptide was observed on the plates supplemented with 7% NaCl. The colony number with 3%, 5%, and 7% NaCl was increased with IPTG compared to the control strain without IPTG in a concentration-dependent manner. The survivability ratio of transformants under the expression of LEA peptide was higher than those not expressing LEA peptide for at all NaCl concentrations tested (Table 1). The number of colony-forming units was highest at 1.0 mM IPTG (Fig. 1 a–c) under NaCl stress at all concentrations. Collectively, these results showed that the expressed LEA peptide conferred salt tolerance to the host cells, but had little effect on 7% NaCl salt

tolerance. Therefore, the function and effects of LEA peptide expression on the growth of the recombinants under high salt tolerance were studied in more detail.

3.2. Temperature tolerance of the expressed LEA peptide in E.coli

The recombinant *E. coli* with the pRSF-Duet LEA plasmid showed greater colony numbers under the 1.0 mM IPTG compared to the control grown without IPTG. LEA peptide increased the growth capacity of the recombinant under both the heat and cold shock treatment compared to the control (Fig. 2).

3.3. Cell viability of recombinant E. coli under salt stress

The rate of WST reduction in the solution (determined by the color change) was linear (Fig. 3a) over a 4 h period, and the amount of dye reduced at $OD_{500\text{-}600}$ generally yielded 2×10^4 cells with a coefficient of variation of replicate wells of 2 to 5%. This demonstrated the utility of the WST assay kit for determining cell viability, and the optimal condition was determined to be a concentration of 2×10^4 cells/well using the WST-dimethyl sulfoxide solution at a final concentration of 0.5 mg/ml with incubation of 4 to 8 h. Using IPTG as an inducer of the pRSF-Duet LEA plasmid showed that expression of the LEA peptide significantly altered the WST reducing ability in recombinant cells. Although cells expressing the LEA peptide showed greater viability under both 5% and 7% NaCl at 4 h compared to the control, viability of the recombinant expressing the LEA peptide was reduced at 8 h under salt stress, reaching the same level as the control (Fig. 3b).

4. Discussion

Several LEA genes have been discovered in plants, and new methods have been developed to study the functions of LEA genes and proteins [20]. The LEA gene has been reported to confer tolerance to stresses such as dehydration[10], drought[7], salt[13], and cold[13] in plants, as well as in microorganisms [21], nematodes [22], collembola [23] and in the crustacean *Artemia franciscana* [24]. LEA proteins can be divided into several different groups based on an amino acid sequence that is characteristic of stress-induced proteins. LEA proteins have attracted particular research interest because they are extremely hydrophilic and are predicted to protect cell functions under stress conditions. S. Hand et al. 2007 and S. Chakrabortee et al. 2007 suggested the physiological roles of LEA protein in the stabilization of sugar groups for protein stabilization via protein–protein interactions, sequestration, and formation of structural networks [24,25]. Such networks have been hypothesized to increase the cellular resistance to physical stresses imposed by desiccation [7, 11, 26]. It is becoming increasingly clear that an ensemble of micro- and macromolecules is an important requirement for stabilizing the cellular condition during exposure to abiotic stress.

To determine the function of LEA peptide, we adopted *E. coli* as a model organism, which is a typical model system for prokaryotic cells. In the present study, we expressed the 11-mer amino acid LEA peptide gene through the pRSF-Duet vector and examined its effects on growth performance under salt, heat, and cold stress. Although we cannot yet elucidate the mechanism of peptide expression in *E. coli*, we previously reported that this LEA peptide exerts its function after translation [16]. Thus, it is possible that the efficiency of translation plays an important role

in effectively expressing this LEA peptide. Indeed, in a previous study, we were able to enhance expression of a target protein through co-expression of this short LEA peptide.

A high salt concentration causes intracellular imbalance and damage to cellular proteins and membranes [27]. Here, we demonstrated that recombinants expressing the short LEA peptide grew and survived better under a high salt concentration and under temperature stress (heat and cold) compared to *E. coli* not expressing the peptide. Similarly, expression of a soybean PM2 LEA protein based polypeptide resulted in enhanced salt tolerance to host cells. The different types of of PM2 polypeptides are contain 262-282 amino acids residues [12]. The PvLEA 22-mer polypeptide takes on a random coil structure in aqueous solution with sodium chloride and potassium chloride, but shows a conformational change in a dry state by forming an alpha-helix, which functions to protect cells against desiccation-induced anti-aggregation [28, 29]. Moreover, PvLEA4 protein was shown to act a molecular shield conferring effective protection against water stress [30] and ultraviolet irradiation [31]. Based on these previous findings and the present results, we hypothesize that the expressed short LEA peptide in the transformant acts as a molecular shield to increase the survivability under abiotic stress, playing a similar role to full LEA proteins and the 22-mer polypeptide. We are currently investigating the specific effects and expression mechanism of the LEA peptide in the transformants under abiotic stress.

Acknowledgements

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Figure legends

Figure 1 Growth performance of *E.*coli under salt tolerance after expression of LEA peptide. The cultures of expressed *E. coli* BL21 (DE3) under different concentration of IPTG were spread on salt contanting LB plates **a)** 3% salt, **b)** 5% salt and **c)** 7% salt. The statistical significance of the difference was confirmed by *t*-test, $^{**}P < 0.01$; $^{***}P < 0.001$. SE±5.

Figure 2. Express LEA peptide response to heat and cold tolerance in *E.coli*. The cultures of expressed *E. coli* BL21 (DE3) under different concentration of IPTG incubate at **a)** 48°C for 30min and **b)** 4°C for 24h were spread on LB plates. The statistical significance of the difference was confirmed by *t*-test, $^{***}P < 0.001$. SE±5.

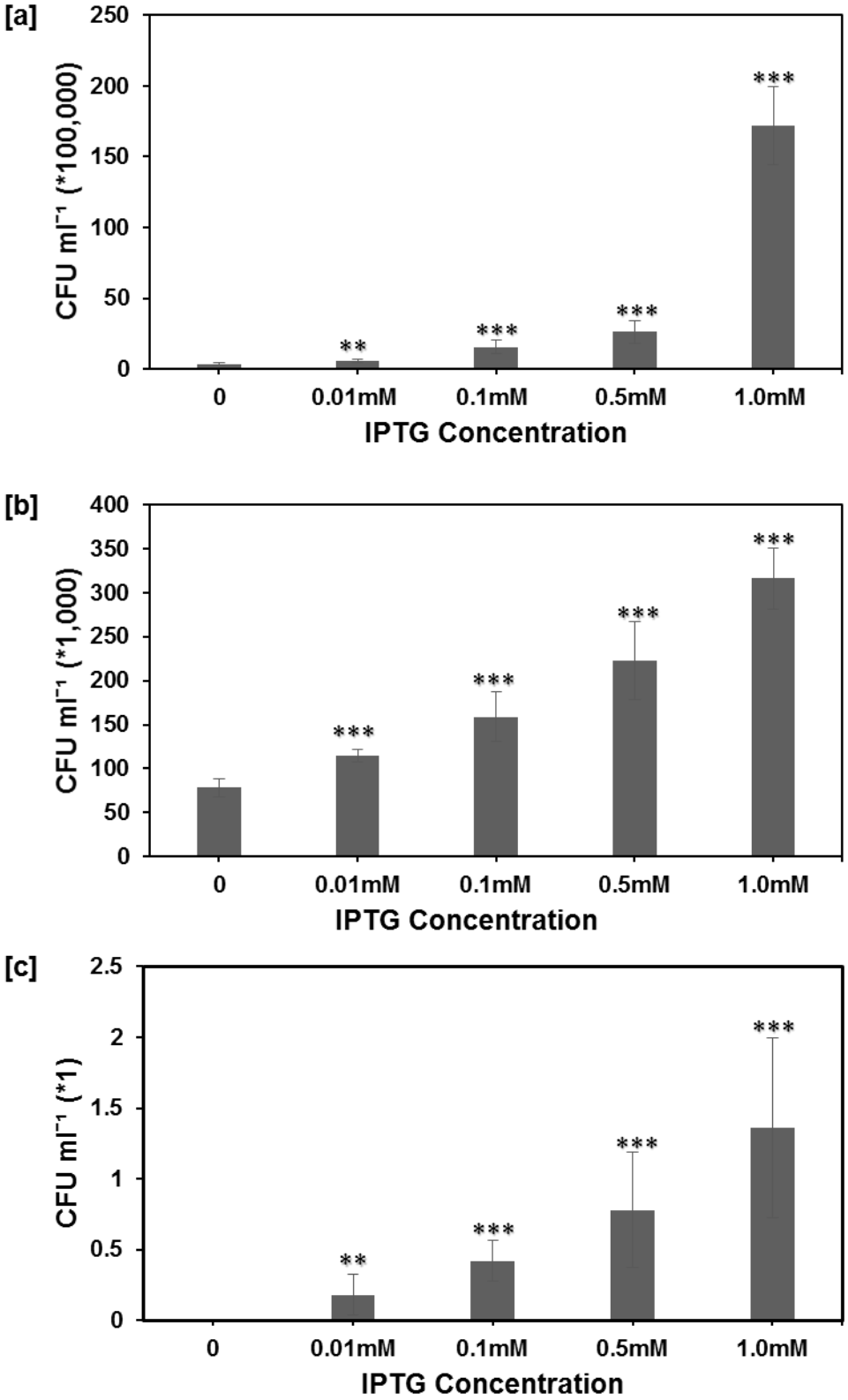
Figure 3. Cell viability of LEA peptide expressed *E.coli* in high salt concentration media. The cultures of *E.coli* BL 21 (DE 3) cell expressed with or without 0.1mM concentration of IPTG transformed in to the 5% and 7% high salt concentration LB media and incubate at 4 h and 8h **a)** Cell growth curves of expressed and unexpressed transfromat at 5% and 7% salt containing LB media. **b)** Cell viability curves of expressed and unexpressed transfromat at 5% and 7% salt containing as described under materials and methods. [**A**] unexpressed LEA peptide transformant in 5% salt LB media, [**B**] expressed LEA peptide transformant in 5% salt LB media, [**C**] unexpressed LEA peptide transformant in 7% salt LB media and [**D**] expressed LEA peptide transformant in 7% salt LB media. SE±5.

Table

Table 1. Survivability ratio of LEA peptide expressed *E. coli* BL21 (DE3) on the plates supplemented with high concentration of salt (NaCl)

Salt concentration	Survivability ratio (%)	
	Without LEA	**With LEA**
3 %	50-200	100-500
5%	10-50	50-70
7%	0	1-2

Figure 1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CFU ml⁻¹ (*100,000)
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200
150
100
50
0
**
***
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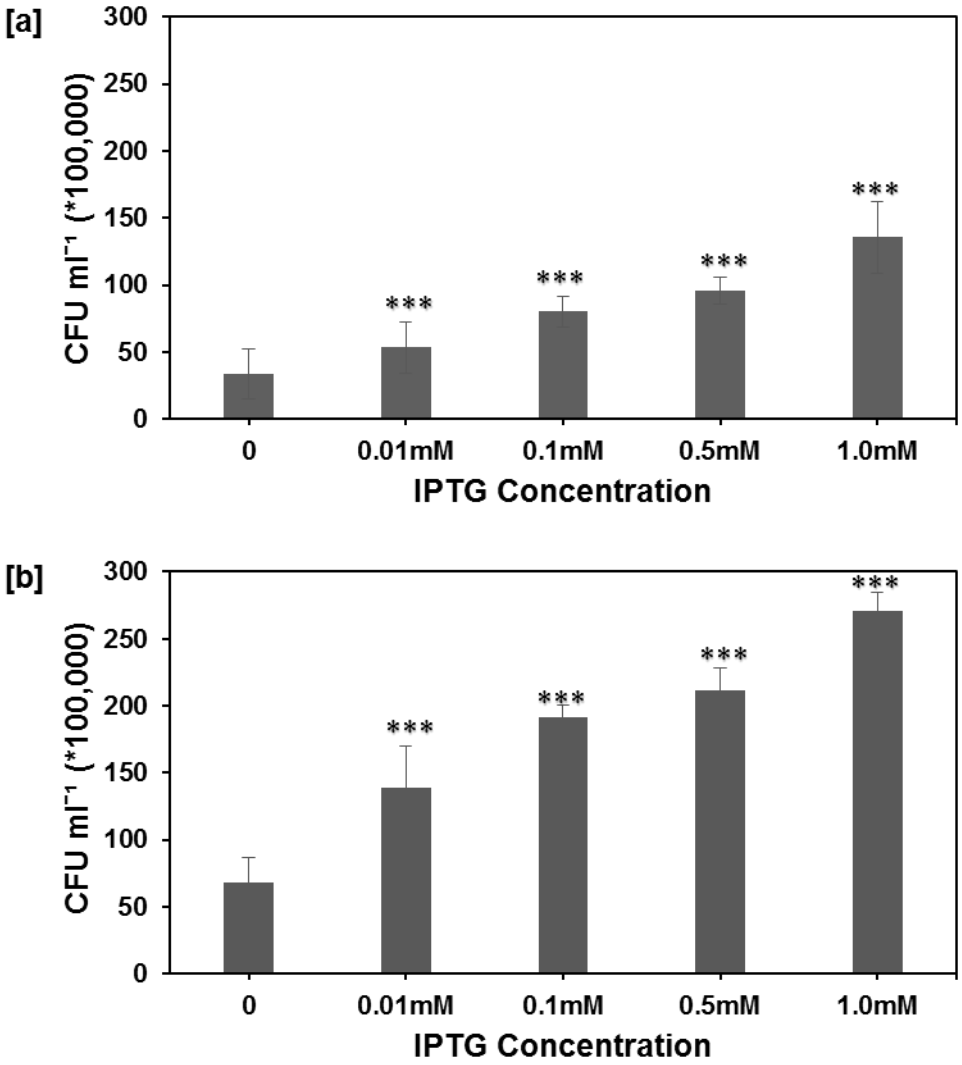
***
0
0.01mM
0.1mM
0.5mM
1.0mM
IPTG Concentration
[b]
CFU ml⁻¹ (*1,000)
400
350
300
250
200
150
100
50
0
***
***
***
***
0
0.01mM
0.1mM
0.5mM
1.0mM
IPTG Concentration
[c]
CFU ml⁻¹ (*1)
2.5
2
1.5
1
0.5
0
**
***
***
***
0
0.01mM
0.1mM
0.5mM
1.0mM
IPTG Concentration

Figure 2


[a]
CFU ml⁻¹ (*100,000)
300
250
200
150
100
50
0
***
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***
0
0.01mM
0.1mM
0.5mM
1.0mM
IPTG Concentration
[b]
CFU ml⁻¹ (*100,000)
300
250
200
150
100
50
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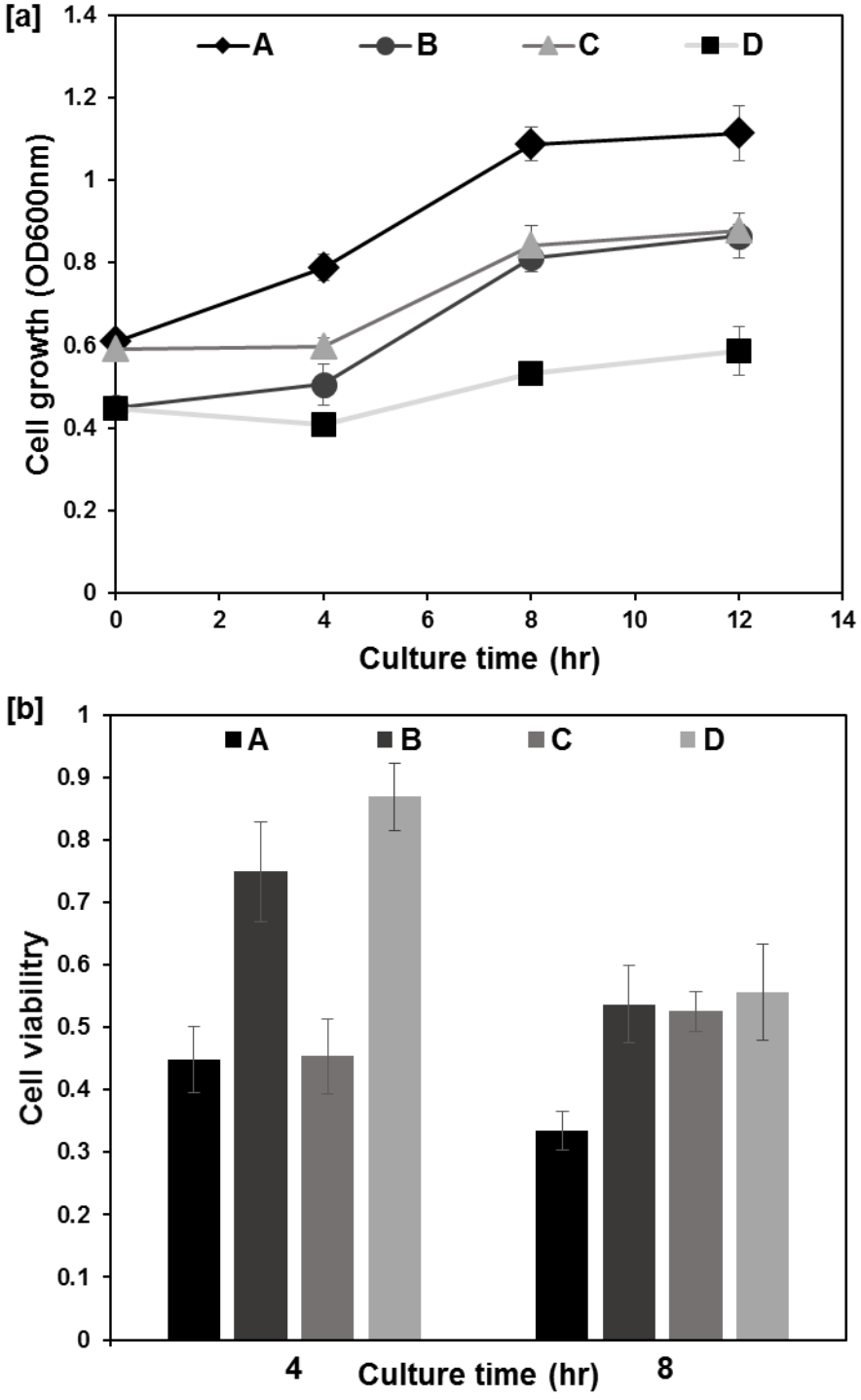
0
0.01mM
0.1mM
0.5mM
1.0mM
IPTG Concentration

Figure 3


[a]
A
B
C
D
Cell growth (OD600nm)
Culture time (hr)
[b]
A
B
C
D
Cell viability
4
Culture time (hr)
8