GroEL–GroES assisted folding of multiple recombinant proteins simultaneously over-expressed in Escherichia coli

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ABSTRACT

Folding of aggregation prone recombinant proteins through co-expression of chaperonin GroEL and GroES has been a popular practice in the effort to optimize preparation of functional protein in Escherichia coli. Considering the demand for functional recombinant protein products, it is desirable to apply the chaperone assisted protein folding strategy for enhancing the yield of properly folded protein. Toward the same direction, it is also worth attempting folding of multiple recombinant proteins simultaneously over-expressed in E. coli through the assistance of co-expressed GroEL–ES. The genesis of this thinking was originated from the fact that cellular GroEL and GroES assist in the folding of several endogenous proteins expressed in the bacterial cell. Here we present the experimental findings from our study on co-expressed GroEL–GroES assisted folding of simultaneously over-expressed proteins maltodextrin glucosidase (MalZ) and yeast mitochondrial aconitase (mAco). Both proteins mentioned here are relatively larger and aggregation prone, mostly form inclusion bodies, and undergo GroEL–ES assisted folding in E. coli cells during over-expression. It has been reported that the relative yield of properly folded functional forms of MalZ and mAco with the exogenous GroEL–ES assistance were comparable with the results when these proteins were overexpressed alone. This observation is quite promising and highlights the fact that GroEL and GroES can assist in the folding of multiple substrate proteins simultaneously when over-expressed in E. coli. This method might be a potential tool for enhanced production of multiple functional recombinant proteins simultaneously in E. coli.

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1. Introduction

The production of recombinant proteins using a variety of host organisms has become essential because of their requirement for various commercial and institutional applications. Although several improved expression hosts are available, Escherichia coli remains a primary choice for recombinant protein production (Busso et al., 2011; Perrakis and Romier, 2008; Romier et al., 2006; Tolia and Joshua-Tor, 2006), due to its low cost nutrition source, minimal laboratory setup, ability to achieve high cell density and availability of information on its genetic and physiological processes (Baneyx and Mujacic, 2004; Gold, 1990; Hockney, 1994; Hodgson, 1993; Makrides, 1996; Olins and Lee, 1993). The protein overproduction processes in E. coli are not without the associated difficulties which include stability and translational efficiency of mRNA, protein degradation catalyzed by host cell proteases, recombinant protein induced toxicity (Makrides, 1996) and problems related to post-translational modification of proteins such as glycosylation and disulfide bond formation. However, the most important problem encountered during protein overproduction is that many recombinant proteins fail to fold into their biologically active conformation in vivo.

The nascent recombinant polypeptide encounters a complex environment in the E. coli cytosol, where there is high macromolecular concentration and restricted physical conditions. Thus, folding of over-expressed protein in such an environment is compromised (Johnson and Craig, 1997). The proper folding of small single domain globular proteins could still be accomplished in such conditions, folding of large, multidomain, multimeric proteins result in kinetically trapped slow folding aggregation prone intermediates (Baneyx and Mujacic, 2004). The incorrectly folded or slowly
folded regions of protein result in prolonged exposure of their hydrophobic regions which remain buried otherwise. This leads to intermolecular association of polypeptide and free interaction with other hydrophobic entities in the cellular environment leading to polypeptide aggregation and eventually inclusion body formation (Banex and Mujacic, 2004; Fahndert et al., 2004; Georgiou and Valax, 1996; Wetzel, 1994). Once inclusion bodies are formed, complex solubilization and refolding procedures must be used to obtain functional proteins (Fahndert et al., 2004). Various techniques for refolding of proteins from inclusion bodies are well documented (Basu et al., 2011; Eiberle and Jungbauer, 2010; Li et al., 2004; Tsumoto et al., 2003; Yamaguchi and Miyazaki, 2014). Tsumoto et al. have reported that the efficiency of refolding of the protein from inclusion bodies significantly depends on the process through which the denaturation concentration is reduced (e.g. one-step dialysis, step-wise dialysis, dilution, etc.) and on the addition of small molecule additives (such as arginine HCl, polyethylene glycol, sugars etc.) in the refolding solvent (Tsumoto et al., 2003).

A recent review focussed on the microfluidic approach in which the solubilized inclusion bodies are passed through laminar flow in a microfluidic chip for reducing the denaturant concentration. This controllable concentration decrease of the denaturant within a short time results in efficient protein refolding (Yamaguchi and Miyazaki, 2014). These methods of optimizing of refolding procedures are time consuming and generally result in significant protein loss (Sorensen and Mortensen, 2005). Therefore, designing advanced strategies to obtain protein in soluble form in vivo could bypass tedious in vitro refolding strategies.

Molecular chaperones and other folding catalysts are well known to facilitate protein folding and maintain protein homeostasis in vivo (Horwich et al., 1993; Johnson and Craig, 1997; Wong and Houry, 2004). These molecules bind to exposed hydrophobic protein sites, thereby preventing non-productive intermolecular interactions (Hartl, 1996; Hartl and Hayer-Hartl, 2002). They catalyze the acquisition of native conformation by allowing an alternative folding pathway through regulated cycles of binding and release of a polypeptide. The most extensively studied chaperones are GroEL and GroES from E. coli (Fenton and Horwich, 1997). During cellular stresses and elevated temperature conditions, the expression of GroEL and GroES increase many fold (Hoffmann and Rinas, 2004a). During recombinant protein overproduction it has been hypothesized that native chaperones can be limiting, resulting in inclusion body formation. However, if chaperones are overexpressed concomitantly with recombinant proteins in vivo then they can help in the preferred folding of recombinant proteins (Georgiou and Valax, 1996; Schlieker et al., 2002). A number of studies on co-expression of the molecular chaperones simultaneous with the recombinant protein in E. coli leads to large quantities of correctly folded protein (Chaudhuri et al., 2008; Gupta et al., 2006; Kyrtatou et al., 2009; Lampaa et al., 2013; O’Reilly et al., 2014; Ray et al., 2012; Sonoda et al., 2010; Thomson et al., 2013; Vonhein et al., 1999; Yanase et al., 2002).

Despite numerous publications reporting the chaperone-dependent contribution to increase soluble target protein in E. coli, no report is found on attempts to fold multiple recombinant proteins over-expressed simultaneously inside E. coli cells with the help of co-expressed chaperone. Many cases report folding of single recombinant proteins with multiple chaperones co-expressed in E. coli (de Marco et al., 2007; Nishihara et al., 1998; Schlapschy et al., 2006) which indicates that cells have potential to produce and process higher amounts of recombinant protein. In one such study, it has been found that co-expression of either GroEL–GroES or DnaK-DnaJ-GrpE alone was unable to prevent CryJ2 aggregation at 30 °C (a major allergen of Japanese cedar pollen). However, with co-expression of both chaperones, the expressed CryJ2 was fully recovered in the soluble fraction (Nishihara et al., 1998). These results indicated that the two chaperones team play synergistic roles in preventing protein aggregation. Schlapschy et al. (2006) have reported that concomitant co-expression of four periplasmic folding catalysts increased the yield of correctly folded retinal binding protein (RBP) by a factor of 4 as compared to the control cells in which RBP was expressed alone in E. coli. A similar effect of multiple chaperone expression on increasing the yield of folded protein has been reported by de Marco et al. where 50 different recombinant proteins were tested for enhancement in their solubility upon the co-overproduction of four chaperone systems GroEL/GroES, DnaK/DnaJ/GrpE, ClpB and the small HSPs HspA/HspB in different combinations. It has been shown that the expression of multiple chaperone systems resulted in an increased solubility of 50% of the proteins tested up to 7-fold (de Marco et al., 2007). Thus, we exploited the E. coli’s capacity to simultaneously over-express multiple recombinant proteins and then fold them using co-expressed chaperones. Based on the available information on their in vivo and in vitro folding ability, two model proteins E. coli maltodextrin glucosidase (MalZ) (Paul et al., 2007) and yeast mitochondrial aconitase (mAcc) (Gupta et al., 2006) have been chosen for this study. These proteins are heavily aggregation prone and refold satisfactorily at the individual level in E. coli while co-expressed with chaperonin GroEL–ES (Gupta et al., 2006; Paul et al., 2007). In this communication we report data using a system in which recombinant chaperones (GroEL–GroES) and target proteins (MalZ and aconitase) were over-expressed using different and independent regulation systems. Parameters like temperature, inducer concentration, duration of induction, etc., which play an important role in enhancing the level of production of the desired proteins in their native form have been optimized. We have studied different aspects of cell growth parameters during the over production of recombinant proteins in the presence and absence of over produced exogenous molecular chaperonin, GroEL–GroES. Our main aim is to improve the production of functional recombinant proteins MalZ and aconitase, simultaneously over-expressed in E. coli, with the assistance of co-expressed chaperonin GroEL and GroES and compare the outcome with chaperone assisted folding of single recombinant protein.

2. Materials and methods

2.1. Materials

Luria broth (LB), Luria agar (LA) and antibiotics were obtained from HiMedia (Mumbai, India). Standard molecular weight markers and isopropyl β-D-thiogalactoside (IPTG) were obtained from Bangalore Genei (Bangalore, India). p-Nitrophenyl-α-D-maltoside (pNPM) was obtained from Merck Biosciences (Darmstadt, Germany). Gel extraction and plasmid purification kits were purchased from Qiagen (Valencia, CA, USA). DNA ligase and other restriction enzymes were obtained from New England Biolabs ( Ipswich, MA, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless noted. The construct pGro7 over expressing GroEL and GroES was generous gift from Dr. Arthur L. Horwich, USA. E. coli strain K12 BL2I (DE3) was used for the expression of various plasmids.

2.2. Cloning of recombinant genes

The genes encoding MalZ and aconitase were cloned in pETDuet-1 vector. pETDuet-1 vector contains two multiple cloning sites (MCS-1 and MCS-2), each under the control of the T7 promoter which allows the simultaneous expression of two recombinant proteins inside the E. coli host. The gene encoding aconitase was cloned in its MCS-2 in Ndel–Xhol restriction sites and malZ gene
Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence 5′-3′</th>
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<tr>
<td>malZ</td>
<td>malZ fw</td>
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<tr>
<td></td>
<td>malZ rev</td>
<td>5′-GATCAGTCTTCATCATTAC3′</td>
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<tr>
<td></td>
<td>aco rev</td>
<td>5′-AAACTCGAGTTTATTCTTCATTCGACCC3′</td>
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was cloned in EcoRI-SacI restriction sites of MCS-1. Sense and antisense primers were designed based on the nucleotide sequence data of malZ and aco, obtained from Gene Bank (NC_006841.1 for malZ and NC_001144.4 for aco) (Table 1). Specific restriction sites for EcoRI and SacI were introduced into malZ forward and reverse primers, respectively. Similarly, NdeI and XhoI restriction sites were incorporated into aco forward and reverse primers. DNA fragments encoding the malZ gene and aco gene were amplified by PCR using their sense and antisense primers. The PCR reaction mixture (25 µl) contained: 1 µg DNA, 0.1 mM dNTPs, 1.5 mM MgCl2, 1 x PCR buffer, 20 pmol of each primers and 1.25 units Taq DNA polymerase. The following conditions were used for PCR amplification: denaturation step; 5 min at 95°C followed by 30 cycles of 1 min at 95°C, 60 s at optimized annealing temperature (52°C for malZ gene and 50.7°C for aco gene) in the annealing step and 2 min at 72°C for the extension step. The resulting PCR product was extended for 10 min at 72°C. The PCR products were analyzed using agarose gel electrophoresis as shown in Fig. 1A.

The PCR purified aco gene was double digested and ligated in NdeI–XhoI sites of pETDuet-1 to form the pETDuet (Aco) clone. pETDuet (MalZ) and pETDuet (MalZ-Aco) clones were obtained by the ligation of double digested malZ gene in EcoRI-SacI sites of pETDuet-1 and pETDuet (Aco) construct. The clone formation was confirmed by restriction digestion of clones with the respective enzymes (Fig. 1B).

2.3. Over-expression of MalZ and aconitase

Over-expression of recombinant proteins, MalZ and aconitase, individually and simultaneously were achieved by transforming competent E. coli BL21 (DE3) cells with pETDuet (MalZ), pETDuet (Aco) and pETDuet (MalZ-Aco) constructs. Transformed E. coli BL21 (DE3) cells were grown in LB medium (containing 50 µg/ml ampicillin) till OD600 reached 0.8–1.0. The cultures were induced with 1 mM IPTG and incubated for 10 h post induction at 37°C with agitation at 220 rpm. A 200 µl cell culture was aliquoted and centrifuged to collect the cell pellet. The cell pellets were resuspended in SDS gel loading buffer, boiled at 100°C and analyzed on 10% SDS-PAGE to confirm protein expression (Laemmli, 1970; Sambrook and Russell, 2001).

2.4. Co-transformation with pGro7

All the above mentioned transformed E. coli BL21 (DE3) cells, harbouring pETDuet (MalZ), pETDuet (Aco) and pETDuet (MalZ-Aco) were made re-competent and were further transformed with pGro7 plasmid. The pGro7 carries genes encoding for GroEL and GroES, confers chloramphenicol resistance and has an arabinose inducible promoter, thus giving a constitutive expression of GroEL and GroES on induction with arabinose. The co-transformed cells were grown in LB medium (containing 50 µg/ml ampicillin and 34 µg/ml chloramphenicol) at 37°C with 220 rpm agitation. Cultures were induced with 1 mM IPTG (for the expression of MalZ and aconitase) and 0.5 mg/ml arabinose (for the expression of GroEL and GroES). The OD600 for the induction was 0.8–1.0. The culture were grown for 12–14 h post induction at 37°C and protein expression was checked on 10% SDS PAGE.

2.5. Determination of the relative intensities of protein bands in the SDS-PAGE gel

A Bio-Rad (USA) gel documentation unit was used for obtaining gel images and its ImageLab software was used for determining the relative quantities of protein bands present on the gel. The ‘Manual frame lanes’ toolbar from the analysis tool box was used for framing the various lanes present on the gel. The drawn lanes were further adjusted to fit the size of the lanes in the gel and lane background subtraction was done to eliminate the background intensity of the gel itself from the bands. Bands were added using the ‘add band’ option and the area of the band was adjusted using the ‘adjust band’ option to optimize the region of the band to be estimated. A relative quantity of the bands selected were measured by selecting a reference band using ‘relative quantity’ option of ‘quantity tools’ from the analysis tool box. The value of intensity obtained from the reference band was considered as unity (or 100%) and all samples were normalized against this value.
2.6. Optimization of parameters for maximum expression of MalZ and aconitase simultaneously over-expressed in the absence and presence of GroEL–GroES

To evaluate the effect of IPTG on recombinant protein production, the E. coli strains transformed with pETDuet (MalZ-Aco) in the absence and presence of pGro7 were grown at 37℃ in LB medium containing respective antibiotics. After OD<sub>600</sub> value reached between 0.8 and 1.0, each culture was aliquoted and distributed into six fractions of 10 ml each. To these fractions, IPTG was added with different concentrations ranging from 0 mM to 1 mM (Christodoulou and Vorgias, 2002). Cultures were incubated overnight at 37℃ at 220 rpm and protein expression level was analyzed by 10% SDS-PAGE (Laemmli, 1970; Sambrook and Russell, 2001). Protein bands were visualized after staining with Coomassie Brilliant Blue R250 (Fig. 2A). Relative band intensities were determined using the gel documentation unit. In this analysis the protein band having maximum value of intensity was selected as reference band whose value was considered as unity and the band intensities of the remaining samples were normalized against it (Fig. 2B).

The time required for maximum expression of MalZ and aconitase after induction with IPTG was determined using the optimized IPTG concentration. Cultures were grown at 26℃ and 37℃ at 220 rpm in the presence and absence of GroEL–GroES. Induction with IPTG was done at OD<sub>600</sub> = ~0.8. At different time intervals, 1 ml aliquots were withdrawn. Samples were normalized for equal number of cells per sample by taking into account their OD<sub>600</sub> values. Collected samples were centrifuged and the pellets were resuspended in SDS loading buffer, heated at 100℃ and analyzed on 10% SDS-PAGE. The protein bands were visualized after staining with Coomassie Brilliant Blue R250 (Fig. 3A). For relative quantification, the band intensity corresponding to the sample collected at 2 h was considered as unity and the band intensities of the remaining samples were normalized against it (Fig. 3B).

2.7. Calculation of specific growth rate constants for recombinant E. coli cells

The BL21(DE3) E. coli strains transformed with various plasmids pETDuet (MalZ), pETDuet (Aco), pETDuet (MalZ-Aco) and
pGro7 were grown at two different temperatures, 26 °C and 37 °C, with and without induction at 220 rpm in shake flasks. At various time intervals, 1 ml of aliquots were withdrawn for turbidity measurements at 600 nm using Beckman UV-Spectrophotometer (USA). Induction was done at OD600 of 0.8–1.0 with 100 μM IPTG for expression of MalZ and aconitate. Expression of proteins was checked by running various samples on 10% SDS-PAGE. E. coli BL21 (DE3) strain was used as a negative control in all these studies. For calculating the specific growth rate constant, \( \mu \), the exponential (or logarithmic) growth phase was used during which the rate of increase of cells is proportional to the number of bacteria present at that time. The specific growth rate constant \( \mu \) was determined using the following equation:

\[
\ln N_t - \ln N_0 = \mu (t-t_0)
\]

where \( N_t \) = number of cells at time ‘t’; \( N_0 \) = number of cells at time ‘t = 0’; \( \mu \) = specific growth rate constant; \( t \) = time in hours.

2.8. Solubility assay of recombinant proteins

For determining the extent of correct folding in vivo, culture broths of different transformed strains expressing MalZ and aconitate at different temperatures were harvested and resuspended in lysis buffer (20 mM sodium phosphate, pH 7.4, Dnase 1 (1 μl/ml), 0.5 mM MgCl₂, 1 mM DTT, 1 mg/ml lysozyme and 1 mM PMSF) (Chaudhuri et al., 2001). The induced cells were exposed to an ultrasonic cell disruptor to release the intracellular components in the lysis buffer. The protocol is based on the assumption that the protein with a native conformation is soluble and physiologically active whereas the misfolded or denatured proteins are insoluble and contain no biological activity (Chaudhuri et al., 2001). Normalization of the cell culture was done by comparing OD600 values such that the same number of cells were taken for the analysis of each sample. The soluble components were separated from the insoluble mass by centrifugation of the cell lysate at 12,000 rpm for 40 min at 4 °C. The supernatant and the pellet were separated, resuspended in the SDS loading buffer and analyzed by SDS-PAGE. The protein bands were visualized after staining with either Coomassie Brilliant Blue R250 or Sypro ruby. Relative quantity of the protein in soluble and pellet fractions was measured by selecting the whole cell extract band as reference band using the ‘quantity tools’ option of the analysis tool box. The biological functionality of both MalZ and aconitate in the supernatant fraction was ascertained by their enzymatic activity assays.

2.9. Aconitase activity assay

Aconitase activity was assayed spectrophotometrically by adding 50 μl of supernatant in a 1 ml reaction volume (90 mM Tris–HCl pH 7.4, containing 20 mM isocitrate dehydrogenase). The formation of cis-aconitate from isocitrate was monitored by measuring absorbance at 240 nm for 10 min at 20 °C using the time/kinetics application on Beckman Coulter DU 800 (USA). The molar absorption coefficient of cis-aconitate is 3.6 mM⁻¹ cm⁻¹ (Kennedy et al., 1983). The enzymatic activity, expressed in units per ml, in each case was calculated from the number of units present in 1 ml of the soluble fraction from the normalized amount of cell pellet.

2.10. MalZ activity assay

MalZ activity was assayed spectrophotometrically based on the reaction in which MalZ acts on p-nitrophenyl-α-maltoside (pNPM) to release p-nitrophenol which absorbs at 405 nm. A 2.5–5 μl aliquot of supernatant was taken and pre-incubated at 37 °C for 10 min in a 900 μl volume of 20 mM sodium phosphate buffer at pH 7.4. A 100 μl addition of p-nitrophenyl-α-maltoside to a final concentration of 0.5 mM was used to start the reaction and allowed to react with sample for 10 min. After a pale yellow colour appeared; the reaction was quenched by adding 500 μl of 1 M Na₂CO₃. The extinction coefficient of p-nitrophenol at 405 nm is 14 mM⁻¹ cm⁻¹ (Tapio et al., 1991). The enzymatic activity, expressed in units per ml was calculated from the number of units present in 1 ml of the soluble fraction from the normalized amount of cell pellet.

3. Results

3.1. Optimization of parameters for maximum expression of MalZ and aconitate simultaneously over-expressed in the absence and presence of GroEL–GroES in E. coli

The recombinant proteins MalZ and aconitate were expressed through IPTG induction from the genes cloned in pETDuet vector under the control of T7 promoter. Fig. 3 summarizes the results on the effect of various concentrations of IPTG on MalZ and aconitate expression. GroEL and GroES were over-expressed constitutively on induction with 0.5 mg/ml arabinose which was added before IPTG induction to ensure abundant supply of chaperones for preventing aggregation and assisting the folding of over-expressed recombinant proteins as soon as they are produced. As shown in Fig. 2, 0.1 mM IPTG was sufficient for optimum expression of MalZ and aconitate, both in the absence and presence of chaperonin. At higher concentration of IPTG the amount of proteins decreased compared to the optimized concentration.

Figs. 3 and 4 show the expression profile for MalZ and aconitate, after induction with IPTG in presence and absence of chaperonin at 37 °C and 26 °C, respectively, as estimated by SDS-PAGE analysis of samples withdrawn at different time intervals. It was found that at 37 °C, MalZ and aconitate expression in absence of chaperonin required 5 h of incubation after induction, whereas, in presence of both GroEL and GroES, expression of recombinant proteins required a longer induction period of 8 h. At 26 °C expression of MalZ and aconitate required 8 h of incubation after induction in the absence of GroEL–ES and 12 h of incubation in the presence of chaperonin.

3.2. In vivo folding of MalZ and aconitate, over-expressed individually vs. simultaneously, in the absence and presence of GroEL–GroES in E. coli

The study of in vivo folding of MalZ and aconitate was carried out while over-expressed individually vs. simultaneously, in the absence and presence of GroEL–ES, at two different temperatures, 37 °C and 26 °C. The extent of correctly folded functional recombinant protein was determined as described in Section 2.8. On disrupting cells and successive fractionation, the soluble proteins appeared in supernatant, whereas, misfolded or aggregated proteins along with cell debris formed the pellet. The SDS-PAGE analysis of the supernatant and pellet fraction of the cell lysates (Fig. 5A and B) showed that when MalZ and aconitate were over-expressed individually, in the absence of exogenous GroEL–GroES at 37 °C approximately 35% of the over-expressed MalZ and ~15% of the over-expressed aconitate was found in the soluble form in the supernatant which increased to ~40% and ~35%, respectively at 26 °C. In the presence of co-expressed recombinant GroEL–GroES approximately 50% of the expressed MalZ was soluble at 37 °C which increased to ~55% at 26 °C and approximately 30% of the expressed aconitate was soluble at 37 °C which increased to ~65% at 26 °C (Fig. 5A and B).
As shown in Fig. 6A and B, when MalZ and aconitase were over-expressed simultaneously in the absence of exogenous GroEL–GroES at 37 °C, approximately 20% of the over-expressed MalZ and ~15% of the over-expressed aconitase was found in the soluble form in the supernatant which increased to ~40% and ~30% respectively at 26 °C. With the assistance of co-expressed recombinant GroEL–GroES approximately 45% of the expressed MalZ was soluble at 37 °C which increased to ~50% at 26 °C and approximately 25% of the expressed aconitase was soluble at 37 °C which increased to ~60% at 26 °C. It thus shows that exogenous chaperones very well assist the folding of MalZ and aconitase when over-expressed simultaneously. A finding comparable with that of their folding when over-expressed individually.

It was demonstrated through activity assays that the soluble form of the proteins while over-expressed individually and simultaneously at 37 °C and 26 °C were functional (Fig. 7A and B). Moreover, at 26 °C in the presence of co-expressed GroEL–ES the activity units obtained for MalZ and aconitase in both instances were comparable (Fig. 7A and B).

![Fig. 4. Optimization of post-induction time for maximum expression of MalZ and aconitase in the absence and presence of GroEL–ES, at 26 °C. (A) 10% SDS-PAGE showing expression level of MalZ and aconitase in absence (top panel) and presence (bottom panel) of GroEL–ES at 26 °C with different duration of incubations after induction. Lane 1 shows medium range molecular weight marker. Lane 2: 0h, Lane 3: 2 h, Lane 4: 4 h, Lane 5: 6 h, Lane 6: 8 h, Lane 7: 10 h, Lane 8: 12 h. (B) Graph shows relative expression level of MalZ and aconitase with different incubation time in the presence and absence of GroEL–ES at 26 °C as depicted through their respective band intensities in the gel. In this analysis, the protein band corresponding to the sample collected at 2 h was selected as reference band whose value was considered as unity and the band intensities of the remaining samples were normalized against it. Error bars represent standard error of the mean (SEM) with mean having 95% confidence limit.

![Fig. 5. In vivo folding of MalZ and aconitase, while over-expressed individually, in the presence and absence of GroEL–ES at 37 °C and 26 °C. 10% SDS-PAGE showing the change in the level of folding of (A) MalZ and (B) aconitase at 37 °C (top panel of gel) and 26 °C (lower panel of gel). The first lane of each panel shows medium range molecular weight marker (M), followed by lanes containing total amount of protein (wc, whole cell), the folded protein (s, supernatant) and aggregated protein (p, pellet) in the absence and presence of GroEL–ES (C) Graph shows the relative percentage of soluble fractions of MalZ and aconitase, in the absence and presence of GroEL–ES, at 37 °C and 26 °C as obtained through their respective band intensities in the gel. Error bars represent standard error of the mean (SEM) with mean having 95% confidence limit.

### 3.3. Growth studies of transformed E. coli cells

On transformation with recombinant plasmids, the growth profile of E. coli cells changes to a large extent as compared to the wild type strain. To study the effect of plasmid characteristics,
IPTG induction and temperature on the growth rate of transformed BL21 (DE3) E. coli cells, various plasmid containing strains were grown with and without induction, at two different temperatures (37°C and 26°C) and their specific growth rates were calculated as described in Section 2.7. The BL21 (DE3) E. coli strain was used as a negative control in all these studies.

The effect of plasmid replication and maintenance on the rate of growth can be judged through the growth profiles of transformed cells without induction. It was found that all the transformed cells without induction showed a decrease on the growth rate as compared to the wild type BL21 (DE3) cells (Table 2). However, at 37°C, the growth rates of all transformants were found to be about 1.5 times higher than at 26°C. To examine the effect of IPTG induction on the growth rate of transformed E. coli cells, induction was carried out with 0.1 mM IPTG at OD_{600} of 0.8–1.0. At 37°C and 26°C, all the transformant expressing either MalZ or aconitase or both.

Fig. 6. In vivo folding of MalZ and aconitase, while over-expressed simultaneously, in the presence and absence of GroEL–ES at 37°C and 26°C. (A) 10% SDS-PAGE showing the change in the level of folding of MalZ and aconitase at 37°C (top panel of gel) and 26°C (lower panel of gel). The first lane of each panel shows medium range molecular weight marker (M), followed by lanes containing total amount of protein (w, whole cell), the folded protein (s, supernatant) and aggregated protein (p, pellet) in the absence and presence of GroEL–ES. (B) Graph shows the relative percentage of soluble fractions of MalZ and aconitase, in the absence and presence of GroEL–ES, at 37°C and 26°C as obtained through their respective band intensities in the gel. Error bars represent standard error of the mean (SEM) with mean having 95% confidence limit.

Fig. 7. Activity assay of MalZ and aconitase, while over-expressed individually v/s simultaneously, in the presence and absence of GroEL–ES at 37°C and 26°C. (A) Graph shows MalZ activity units (units/ml of soluble protein) obtained in the absence and presence of GroEL–ES at 37°C (light gray bars) and 26°C (dark gray bars) while over-expressed alone v/s co-expressed with aconitase. (B) Graph shows aconitase activity units (units/ml of soluble protein) obtained in the absence and presence of GroEL–ES at 37°C (light gray bars) and 26°C (dark gray bars) while over-expressed alone v/s co-expressed with MalZ. Error bars represent standard error of the mean (SEM) with mean having 95% confidence limit.

Table 2 Specific growth rate constants (μ) for the growth of BL21 (DE3) E. coli strain under various plasmid-containing situations at 37°C and 26°C.

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<th>E. coli strain</th>
<th>μ at 37°C (h⁻¹)</th>
<th>μ at 26°C (h⁻¹)</th>
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<tr>
<td>BL21 (DE3)</td>
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<td>0.14</td>
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<tr>
<td>BL21 (DE3) + MalZ</td>
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<td>0.11</td>
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<td>BL21 (DE3) + Aco</td>
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<td>0.15</td>
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<td>BL21 (DE3) + GroEL–ES</td>
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</tr>
<tr>
<td>BL21 (DE3) + MalZ + GroEL–ES</td>
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<td>0.16</td>
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<tr>
<td>BL21 (DE3) + Aco + GroEL–ES</td>
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<td>0.17</td>
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<td>BL21 (DE3) + MalZ + Aco</td>
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<tr>
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either pETDuet (MalZ) or pETDuet (Aco), both in the presence and absence of GroEL–ES. Thus, the growth of cells was not affected with the simultaneous over-expression of both proteins and further supported that chaperones were able to act in a similar way in both the cases.

4. Discussion

It is common practice to use multiple co-expressed chaperones to fold individual recombinant proteins in *E. coli* (de Marco et al., 2007; Nishihara et al., 1998; Schlapchzy et al., 2006). Cells probably do have potential to produce and process higher amounts of recombinant proteins in their functional state. Due to versatile usage of recombinant proteins, cost effective and simpler protocols for their production are always sought after. Considering these facts, it was decided to produce multiple recombinant proteins simultaneously in *E. coli* with the assistance of a single co-expressed chaperone system. In concurrence to this effort, the first attempt has been made to use one of the well studied chaperone system GroEL–GroES to explore its ability to properly fold two strongly aggregation prone recombinant proteins, MalZ and aconitase, over-expressed simultaneously in BL21 (DE3) *E. coli* cells.

It is well known that recombinant protein production stimulates stress responses in the bacterial cell and these cells are not able to react appropriately to such situations due to metabolic limitations. This stress during recombinant protein production can prevent the over-production of several recombinant proteins and also hampers the quality and quantity of recombinant product (Hoffmann and Rinus, 2004b). Another challenging task for multiple recombinant protein production is to find plasmids which are compatible with their origin of replication and antibiotic resistance so as to express the recombinant proteins efficiently. Also, the problem usually encountered while expressing recombinant proteins through multiple plasmids is uneven and reduced expression of proteins because of maintenance of less copies of each plasmid (Tolia and Joshua-Tor, 2006). Therefore, to obtain good yield of recombinant proteins it is pre-requisite to reduce production stress either by optimizing cloning and expression conditions or chaperone co-expression or both.

We developed a two vector based system for expressing two aggregation prone recombinant proteins simultaneously along with a molecular chaperone. One vector, under IPTG regulation, was used for the simultaneous over-expression of two large aggregation prone recombinant proteins MalZ and aconitase and another, arabinose-inducible, was used for the co-expression of chaperonin GroEL and GroES. This is certainly a feasible way for the independent induction and maintenance of the level of expression of both chaperones and target recombinant proteins.

After successive cloning, controlling parameters such as concentration of inducer, duration and temperature of induction were optimized for achieving a higher level of expression for the selected recombinant proteins MalZ and aconitase. It was found that the yield and solubility of proteins were affected by these factors. The expression of recombinant proteins in *E. coli* can be controlled by using different concentrations of the inducer. In this case a concentration of 0.1 mM IPTG resulted in higher quantities of MalZ and aconitase expression which suggests gentle induction allows for better folding by inducing protein expression at a slower rate.

The post-incubation time required to over-express MalZ and aconitase in the absence of chaperonin was found to be less compared to the time required in presence of the complete GroEL–GroES folding machinery. The reasoning underlying this observation is that since protein synthesis is an energy driven process and in the presence of exogenous GroEL–ES, the cell utilizes its energy in the form of ATP hydrolysis for correct folding of proteins (Chaudhuri et al., 2001; Farr et al., 2003; Richardson et al., 1998) along with host protein synthesis. In the presence of chaperonin a significant reduction in the rate of protein synthesis occurs leading to a longer induction period to over-express recombinant proteins. Post-induction incubation time to over-express MalZ and aconitase at 26 °C was longer as compared to 37 °C, which suggests that the rate of protein synthesis is low at lower temperature thus requiring a longer time for complete induction to occur (Fink, 1998). Optimization of induction time is crucial since shorter post-induction period may result in the reduction of protein over-expression and a longer post-induction interval, although enhances protein expression, may also induce proteolysis of the target proteins, again leading to the reduction in the protein yield.

In the present study it was found that the co-expressed chaperones (GroEL–GroES) elevated the soluble active folding percentage of over-expressed recombinant proteins (MalZ and aconitase) at both temperatures, 37 °C and 26 °C, and in both cases of single and double recombinant protein expression. As suggested by activity assays, for both cases, the maximum percentage of correctly folded functional MalZ and aconitase were obtained at 26 °C when exogenous GroEL–GroES were co-expressed. The solubility and productivity of the expressed recombinant protein is largely affected by temperature. At higher temperature there is more protein synthesis as compared to lower temperature, but lower temperature provides better folding of the protein. At lower temperatures inclusion body formation is substantially reduced and hydrophobic interactions decrease between nascent polypeptides which bring about a reduction in the rate of aggregation (Fink, 1998). At higher temperature, the rate of the aggregation reaction is increased due to strong temperature-dependence of hydrophobic interactions (Miot and Beton, 2004). As a result of competition between aggregation and the chaperone assisted folding reaction, loss of the newly synthesized protein takes place which leads to the formation of inclusion bodies. With exogenous chaperone assistance the folding of MalZ and aconitase, when over-expressed simultaneously, was comparable with that of their folding when they were over-expressed individually, which strongly suggests that GroEL–GroES do have potential to properly fold and process multiple target proteins in *E. coli* cells.

Cells efficiently utilize energy to carry out essential functions required for its survival. The distribution of energy is well defined and among all the processes performed, protein synthesis is the most energy consuming process. When the cell is forced to over-produce recombinant proteins not necessarily required for its survival, it experiences an additional burden in terms of the energy utilization for the maintenance and expression of recombinant proteins. This disturbs the intrinsic energetics of the cell and hence directly affects its rate of growth (Glick, 1995). It is reported that the presence of a large number of recombinant plasmids in a cell reduces its specific growth rate (Kobayashi et al., 2002; Lee and Moon, 2003). In the present study, we have observed that at both temperatures (37 °C and 26 °C) the transformed cells without induction showed an overall decrease in their growth rate (compared to the control) (Table 2). However, at 37 °C, the growth rate of all transformants was more than that at 26 °C. It may be anticipated that at higher temperature, enhanced production of endogenous GroEL–GroES occurs which results in correct folding of various proteins in the cell thereby preventing their aggregation. This essentially increases the efficiency of the cell and in turn causes enhancement in its growth rate. In *E. coli* such an increase in production of heat shock proteins at higher temperatures has been reported (Kusukawa and Yura, 1988; Rosen and Ron, 2002; Yura et al., 1993).

Some varying trends were observed on induction with IPTG at both temperatures in the correlation of growth rate vs. presence and absence of pGro7 plasmid in *E. coli* cells involved in
recombinant protein production. All the transformant categories expressing recombinant proteins (without recombinant GroEL/ES) showed a reduction in the growth rate (compared to the control), whereas, with recombinant GroEL–ES co-expression, there occurred an increase in the growth rate of all the transformed cells. It is reported that IPTG causes a burden to the cells as uptake of IPTG involves the use of proton pumps (Krzewinski et al., 1995; Seol and Shatkin, 1992) which in turn may influence the growth rate of the cells. Another factor is that IPTG cannot be degraded inside the cell and expressing recombinant proteins through IPTG induction may slow the growth rate of E. coli. The effect of IPTG on the growth rate of recombinant protein producing E. coli cells has been reported (Malakar and Venkatesh, 2012). Previous research demonstrates that the reason for the reduction in the growth rate of the cells expressing recombinant proteins (without recombinant GroEL/ES) upon induction with IPTG could be above mentioned inducer effect. However, on inducing the cells carrying pGro7C plasmid, the cells start producing recombinant GroEL–ES along with their endoge- nous counterpart. The exogenous chaperones may help a fraction of the over-expressed proteins to fold correctly, which in turn enhances the cell efficiency by minimizing the toxic effects of protein aggregation resulting in its enhanced growth rate. Hence, on induction, all recombinant protein over-expressing strains which were co-expressing recombinant GroEL–ES showed an increase in the growth rate by subsiding the inducer effect.

Another significant observation was that upon induction at both temperatures the specific growth rate of the E. coli cells harbouring pETDuet (MalZ-Aco) plasmid was comparable with that of the specific growth rate of cells harbouring either pETDuet (MalZ) or pETDuet (Aco), both in the presence and absence of chaperones. Since the specific growth rate has been regarded to be the intrinsic variable affecting recombinant protein synthesis it means that the growth of the cells was not hampered with the simultaneous over-expression of MalZ and aconitate and further supported that chaperones were able to fold both proteins correctly and in soluble form.

It may be concluded that with the assistance of co-expressed GroEL–GroES, it is possible to efficiently fold multiple aggregation-prone recombinant proteins, MalZ and aconitate, over-expressed simultaneously in E. coli cells. This observation indicates that the cells do have potential to over-express and fold multiple functional recombinant proteins with the assistance from co-expressed molecular chaperone. It is worth applying this technique of multiple protein folding inside E. coli cells using co-expressed GroEL–ES assistance on various other proteins of choice and find out whether GroEL–ES can also fold other proteins over-expressed simultaneously in the E. coli expression system. It is also reasonable to explore multiple protein folding processes in E. coli using other chaperone systems. The process of chaperone assisted simultaneous folding of multiple recombinant proteins in E. coli may be helpful in a fermentation bioreactor to achieve a higher quantity of functional protein. Since the culture conditions can be accurately controlled in a bioreactor the fermentation culture may be utilized to test the efficiency of GroEL assisted folding of multiple recombinant proteins in E. coli.

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