International Journal of Applied and
Natural Sciences (IJANS)
ISSN (P): 2319–4014; ISSN (E): 2319–4022
Vol. 9, Issue 4, Jun–Jul 2020; 63–72

International Academy of Science,
Engineering and Technology
Connecting Researchers; Nurturing Innovations

PROTEIN EXPRESSION AND PURIFICATION OF A MOLECULAR CHAPERON IN ASSOCIATION WITH HEAT STRESS TOLERANCE IN BACILLUS SUBTILIS (D18) ISOLATED FROM HOT SPRING OF INDIA

Dushyant Pratap Singh[1], Ikram Qureshi[2] & Kanika Kumar[3]

[1,2]JJT University, Rajasthan, India

[3]ICAR-National Research Centre on Plant Biotechnology, LBS Centre, IARI, New Delhi, India

ABSTRACT

A thermo tolerant bacterial strain was isolated from hot spring of Sohna, Haryana, India, classified and named as B.subtilisD18 after morphological and 16S rRNA gene sequence analysis. This isolate was used to amplify groEL gene, which encodes molecular chaperon GroEL. The full length gene was 1.6 kb in length encoding a polypeptide of 108amino acid residues. The calculated molecular weight and pI of the protein were nearly 60 kDa and 4.75, respectively. The amino acid sequence of the gene was similar to other groEL proteins and the homologous groEL of different microorganisms. The groEL gene of B. subtilis was successfully expressed in Escherichia coliBL21 (DE3) strain using pET expression systems and purified by polyhistidine tag using Ni-NTA (nitrilotriacetic acid) resin column. Heterologous expression of groEL of B.subtilis in E. coli BL21(DE3) allows the growth of E.coliup to 42°C for 16 h, suggesting that groEL from B.subtilis imparts tolerance to host cells under elevated temperatures.

KEYWORDS: *Bacillus Subtilis, Molecular Chaperone, Expression Vector, Heterogonous Expression, His-Tag Purification*

Article History

***Received:* 14 Jun 2020 | *Revised:* 17 Jun 2020 | *Accepted:* 26 Jun 2020**

INTRODUCTION

The major problem to any cell by heat shock is the immediate formation of denatured and misfolded proteins, often referred as nonnative polypeptides, which tend to aggregate (Schumann 2003). Eukaryotic cells when exposed to stress conditions responds by synthesize specific set of HSP's (Shermanet al 1992).Study conducted by Du Tran et al (2015) suggested that chaperones may have protective role in cells facing environmental stress. Molecular chaperones protect newly synthesized or stress-denatured polypeptides from misfolding and aggregation in the highly crowded cellular environment (Susin et al 2006). The major molecular chaperones in bacteria include DnaK machine and the GroE machine (GroEL and GroES) as described in De Carolis et al (2011). Out of many proteins which were up-regulated at 37°C it was found that an important role is played by molecular chaperone groEL in assisting the protein folding and unfolding under both normal and stress conditions (Kunst et al 1997). The well-studied Escherichia coli chaperonin GroEL binds non-native substrates and encapsulates them in the cavity, thereby sequestering the substrates from unfavorable conditions and allowing the substrates to fold (Kumar et al 2015). The heat shock response is an important adaptation enhancing survival of organisms at high temperature in which a battery of genes is activated by heat. Hot springs are the natural habitat for a

wide range of thermophilic and hyperthermophilic microorganisms which have optimal growth temperatures of > 55°C and > 80 °C, respectively. A bacterial strain of Bacillus subtilis D18 was isolated and purified from collected water samples of a hot spring. B. subtilis is an aerobic, endospore-forming, rod-shaped bacterium commonly found in soil, water and in association with plants. B.subtilis and his associates are an essential source of industrial enzymes and their capacity to secrete such enzymes is the cause of much commercial interest in those bacteria.

MATERIAL AND METHODS

Bacterial Strain and Cloning of Gene

The Bacillus subtilisD18 thermo tolerant has been isolated from the water of the Sohna hot sulphur water, which is situated inside the Shiva temple in the Haryana State of India. Gene groEL was amplified and cloned in pDrive TA vector yielding pDEL (pDrive-groEL). After digestion of pDEL with BamHI and SalI, excised groEL gene fragment was cloned into the corresponding sites of pET-28a, yielding the recombinant expression vector pTEL (pET28a-groEL). Then E. Coli BL21 (DE3) cells were transformed with recombinant plasmid pTEL.

Spot Test for Stress Tolerance

Chaperones can function in increasing the yield of correctly folded, soluble, active proteins both in vitro and in vivo resulting in involvement in stress tolerance (Amrein et al 1995). To analyse this statement spot assays were carried out in luria agar using rectangular petri plates against two different stress (300 mM NaCl and 300 mM Mannitol) Yadav et al (2012). Transformed BL21 (DE3) cells (both pTEL: BL21 and pET28a:BL21) were allowed to grow in Luria broth until OD_{600} reaches 0.6. For induction, 1 mM IPTG was added to 0.6 OD cells and were allowed to grow for 12 h at 37°C with constant shaking (180 rpm) in an incubator shaker. Next day, cultures were diluted up to three dilutions 10^{-4}, 10^{-5} and 10^{-6} cells (Vorderwülbecke et al 2004). 5 µl from each dilution was spotted on plain LB agar plates as well as LB agar plates, supplemented with 300 mM NaCl and 300 mM Mannitol. Plates were incubated at 37°C for 12 hr.

Temperature Stress Tolerance Test

To determine the role of groEL in heat stress, another spot assay was performed at two different temperature stresses. Plates were prepared exactly same as above. After dilutions 10^{-4}, 10^{-5} and 10^{-6} spots were placed on LB agar plates, allowed to dry in laminar flow for 20 minutes and subjected to stress at two different temperatures 37°C and 42°C for 16 hours. All the plates were kept inside a humid chamber to prevent the rapid drying of the medium (Aggarwal et al 2012).

Assay for Temperature Tolerance in Liquid Culture

Functional analysis of the gene pET28a: groEL in BL21 (DE3) and pET28a in BL21 was also tested in plain LB liquid culture as well as LB supplemented with sodium chloride and mannitol, separately. *E. coli* BL21 (DE3) cells transformed with recombinant plasmid and vector alone were grown as same as for spot assay, diluted to 0.6 OD_{600} and 400 µl culture was inoculated in 10 ml LB medium containing 300 mM NaCl and 300 mM mannitol, and incubated at 37°C (Yadav et al 2012). OD_{600} was measured by harvesting the cells at every 2 h till 12 h.

Protein Expression and Purification of the GroEL

Recombinant E.coli cells (BL21-DE3: pTEL) were inoculated in 10 ml LB having antibiotic (Kanamycin 40mg / ml) and incubated overnight at 37°C with constant shaking at 180rpm in an incubator shaker. Cells from overnight inoculum were inoculated in fresh 10 ml LB and incubated at 37°C until OD_{600} reaches 0.6, calculated by observing the collected fractions

in spectrophotometer (Amrein et al 1995). The recombinant protein was expressed by adding 1 mM IPTG (Isopropyl β-D-thiogalactoside) at 0.6 OD_{600} and fractions were collected after time intervals of 1 hr, 2 hr and 3 hr from induction and were kept on ice. PET28a in BL21 (DE3) was also induced with 1mM IPTG and fractions were collected. Cells were harvested at 4°C in a refrigerated centrifuge (eppendorf) and the pellets were dissolved in chilled lysis buffer (50mM tris-cl and 5mM DTT, pH 7.5) for sonication. Cell wet weight to buffer volume of 1:5 was used to dissolve the pellet. All sonication steps were performed in ice by giving 10 sec pulse followed by 30 sec cooling to the dissolved pellet. To quantify GroEL levels at different time intervals, the same amounts of total lysate (1-20 µg / lane) were subjected to sodium dodecyl sulfate- polyacrylamide gel (14 % resolving and 4 % stacking gel) electrophoresis (SDS-PAGE). GroEL expression levels at different time periods were directly quantified from the (CBB-250) Coomassie brilliant blue-stained SDS gel. Induced GroEL protein was purified using Ni-NTA column by binding the protein to the column with binding buffer and washing unwanted proteins with different elution buffers (Sodium phosphate buffer, NaCl and Imidazole).

RESULTS AND DISCUSSIONS

Amplification of GroEL

Molecular chaperone groEL was amplified from the genomic DNA of B.subtilisD18.Thermoprime plus taq DNA polymerase was used for the amplification which has 5' to 3' polymerization and exonuclease activity. 100µl reaction volume was used to elute the gene fragment having 3'-An overhang and cloned in pDrive TA cloning vector (Quigen) by transforming E.coli DH5α competent cells (pDEL). Randomly six clones were picked and confirmed for the presence of desired gene fragment by restriction digestion with BamHI and SalI. Two fragments were released from positive clones after restriction digestion, one of gene 1635 bp and another of vector 3.8 kb (3851bp). Nucleotide sequencing was outsourced with gene specific primers both from 5’ and 3’ ends for a single plasmid. Interpretation of sequencing results after ncbi BLAST analysis shows 99 % homology with groEL gene of Bacillus subtilis (GenBank CP029461.1: Bacillus subtilis gene, E Value=0.0 with 100 % query coverage and 97 % identity) where ‘E’ value describes the random background noise and decreases exponentially as the homology match increases.

Cloning in Expression Vector

From pDEL (pDrive: GroEL) construct, gene fragment was excised again by digestion with BamHI and SalI restriction enzymes and legated again with pET28a expression vector (already digested with same enzymes). Electro-competent cells of E. coli BL21 (DE3) cells were prepared and transformed with recombinant plasmid pTEL (pET28a-groEL) and intact pET28a vector alone for comparative analysis of expression. Random six clones which were positive after kanamycin (40mg / ml) selection was confirmed for the presence of Desired insert by restriction digestion with BamH1 and SalI after plasmid isolation. After overnight digestion reaction, mixtures were subjected to electrophoresis, which unveils the presence of insert 1635bp along with pET28a band ≈5.3kb (Guglielmi et al 1991).

Spot Test Analysis

Recombinant and control cells were both found on LB agar as well as on an enriched medium of 300 mM NaCl and 300 mM mannitol. After 12 hours of incubation, it turns out that recombinant cells work better than control cells (Figure 1). In all dilutions in recombinant cells, colonies were significantly higher than vector control. It demonstrates that cells with a recombinant gene do better even under stress conditions at dilution 10^{-6}.

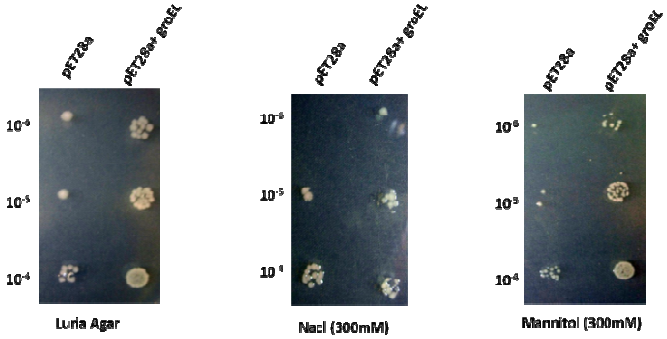


Figure 1: Comparison of pET28a and pET28a + groEL at Different Salt Stress.

Temperature Stress

After the cells are subjected to temperature stress in a recombinant gene groEL at 42° C for 12 hours, cell growth is better demonstrated than vector control cells at the two temperatures. In terms of control cells, combined cells work at all 37°C and 42°C temperatures and at all three dilutions (Figure 2). The figure shows that the effect of dilution 10^{-6} at 42°C is more efficient when the temperature increases.

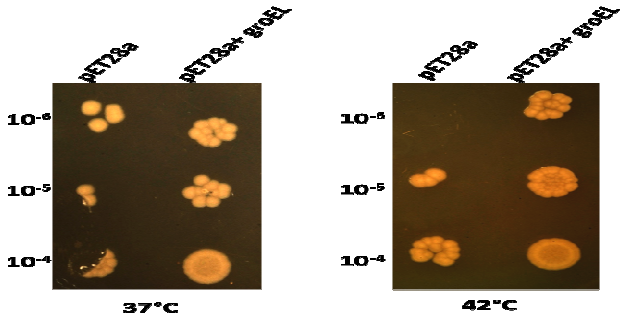
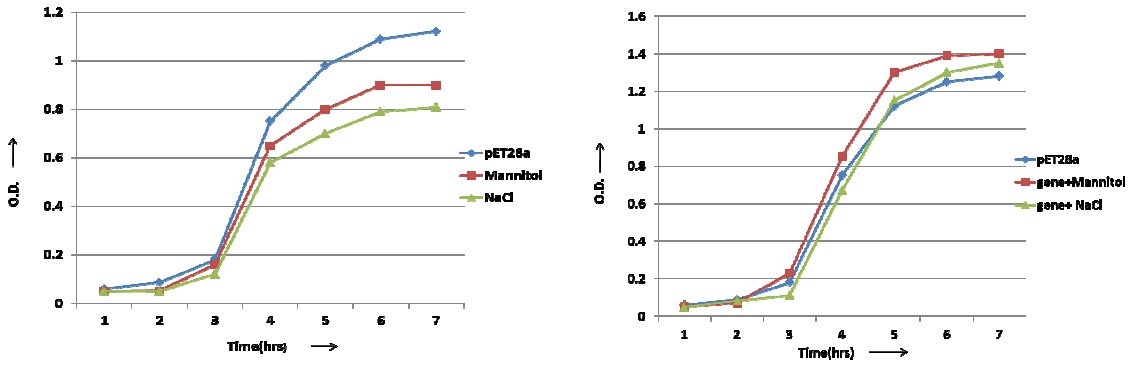


Figure 2: Growth Comparison of pET28a and pET28a+groEL at Different Temperatures.

Liquid Culture Test Analysis

Analysis of growth pattern by measuring OD shows almost equal number of cells in all three cultures, pET28a:BL21, pTEL: BL21 in NaCl and pTEL in Mannitol after 2 hrs of incubation at 37°C. However, as the period increases pTEL: BL21 growth rate decreases with NaCl, it demonstrates cell counts inhibition after 4 hr of incubation, and pTEL: BL21 displays higher cell counts than the vector alone in mannitol. Recombinant cells tend to increase even after 10 hours in terms of vector regulation. (Figure 3)



A **B**

Figure 3: A. Growth Curve of pET28a With and Without Salt Stress. B. Growth Curve of Recombinant Clone After Induction.

Protein Expression Analysis of GroEL

The expression vector pET28a was transformed to Ecoli BL21 (DE3) in the LB medium, and induced by IPTG at 0.6 OD600 to investigate the expression of groEL in heterogonous host. Upon 2 hours of treatment, the recombinant protein has been induced. Both unindicted and induced fractions of protein (2h, 4h and 6h) have been obtained after sonication, and subjected to SDS-PAGE along with an un induced and inducted vector with a multicolored protein marker (10-260 kDa, wide spectrum). GroEL ORF consists of 1635 nucleotides and encodes a protein with an average molecular weight of 60 kDa of 544 amino acids (W. Li et al 2008) (Figure 7). After distaining the gel, presence of 60 kDa band in all three induced fractions of pTEL: BL21 (DE3) confirms that protein was expressed successfully after IPTG induction, whereas the band was absent in both induced and un induced pET28a:BL21DE3. This data further confirmed that no protein was formed in pET28a:BL21DE3, at the same time desired gene groEL was inserted and expressed properly into host cells.

His Tag Purification of Protein

For the purification of desired protein having His Tag, 1ml His SpinTrap™ columns were used. Standard micro-centrifuge was used for elution of protein from His SpinTrap. Columns were placed in a 2 ml micro centrifuge tube to collect the liquid during centrifugation. 600µl clear lysate was added to the column directly to allow binding for 2 mins. After binding, column was washed and eluted with 200µl elution buffer in a clear tube. First elute contains the majority of protein. Obtained protein is free from other unwanted proteins and was of desired size 60kDa (Figure 4)

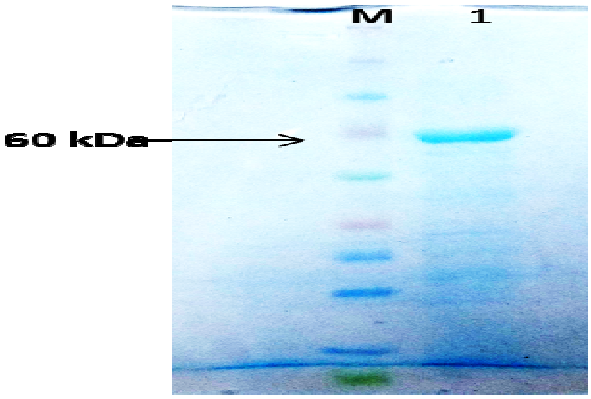


Figure 4: Purified Protein Lane M: Multicolor Protein Marker, Lane1: Purified 60kDa Protein.

In-Silico Analysis of Peptide

Tools for in silico analysis of protein are of major significance for making use of data for development of drug and health care. Proteins are complex macromolecules, which have major role in performing cellular activity. Thus, analysis and knowledge of protein is important for determining the protein function and to understand their role and mechanism.

Peptide Prediction

Expasy translate tool is used to translate 1635 nucleic acid sequence to their corresponding 544 peptide sequences.

Figure 5

Prediction of ORF

Open reading frame was predicted based on the availability of the template sequence. Free read frames (ORFs) are read frame elements that do not include stop codons. A reading frame can be the nuclear triplets that can be read as codons specified in amino acids. There are 3 possible reading frames on one strand of a deoxyribonucleic acid sequence. In the deoxyribo-nucleic acid chain, long ORFs may suggest nominee macromolecular coding regions. The candidate protein coding regions in a DNA sequence may be indicated by long ORFs. The open reading frame is the portion of a reading frame that can be translated. The search parameters for the ORF finder were: minimum length of ORF was selected as 150, genetic code selected was archaeal and bacterial, ATG was used as start codon. Based on these parameters ORF Finder predicts that the amplified sequence comprises a full ORF forming a 544 residue peptide.

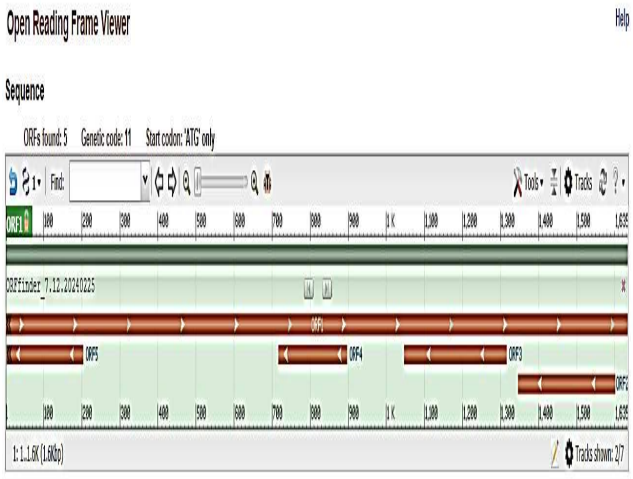


Figure 6

lark subset... Marked: 0 Download marked set as Protein FASTA

Label	Strand	Frame	Start	Stop	Length (nt | aa)
ORF1	+	1	<1	1635	1635 | 544
ORF4	-	2	1316	1047	270 | 89
ORF3	-	2	1601	1347	255 | 84

Figure 7: Amino Acid Sequence and ORF of GroEL.

Validation of Predicted Model by Ramachandran Plot

PROCHECK is a tool to substantiate the spatial arrangement (stereochemistry) of protein structures. The plot generated by this program is Ramachandran plot, which is a plot of phi-psi torsional angles where darkest region is the most favored region consisting of most of the residues. Plot analysis shows that 70 % of all residues of groEL are in the most favored regions (AB L). 9.4 % of residues are in the most allowed regions and 0.6 % of residues are found in the generously allowed region and 0.0 in the disallowed region. The plot was generated using RAMPAGE program. Number of glycine residues are 26 and proline are 15. Total number of observed residues is 205.

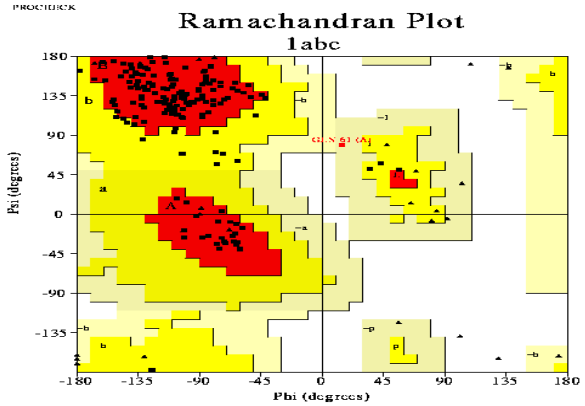


Figure 8: General Amino Acid Representation of Ramachandran Plot for the Modeling of GroEL.

CONCLUSIONS

Chaperonins are large oligomeric double ring assemblies that carry out an essential function in the cell, assisting many newly translated proteins to fold to their native forms. Over the last decade, it has been well established that GroEL / GroES are involved in driving protein folding under conditions, where spontaneous folding simply does not occur. In another study, comparison between DnaK and GroES / GroEL shows that DnaK is not required for growth of Bacillus subtilis at temperatures ranging from 16°C to 52°C, whereas GroEL is essential both at normal temperatures and during heat stress GroEL ensures the correct folding of proteins in an ATP-regulated manner under normal growth conditions and under conditions of stress. This study also concludes that groEL is an essential and decisive factor when micro-organisms are facing heat stresses in extreme environments. According to performed experiments, ambient temperature for E.coli is 37°C, but when it is transformed with groEL, it performs better at 42°C and for more duration under stress conditions. Which concludes that, it must be a gene responsible for thermo tolerance under stress conditions?

REFERENCES

1. *Schumann, W. (2003). The Bacillus subtilis heat shock stimulon. Cell stress & chaperones, 8(3), 207217.*
2. *Sherman, M. Y., & Goldberg, A. L. (1992). Heat shock in Escherichia coli alters the protein-binding properties of the chaperonin groEL by inducing its phosphorylation. Nature, 357(6374), 167.*
3. *Du Tran, C. T., Bernard, C., & Comte, K. (2015). Cloning of some heat shock proteins genes for further transcriptional study of Planktothrix agardhii exposed to abiotic stress. Folia microbiologica, 60(4), 317323.*
4. *Susin, M. F., Baldini, R. L., Gueiros-Filho, F., & Gomes, S. L. (2006). GroES / GroEL and DnaK / DnaJ have distinct roles in stress responses and during cell cycle progression in Caulobacter crescentus. Journal of bacteriology, 188(23), 80448053.*
5. *De Carolis, E., Posteraro, B., Florio, A. R., Colonna, B., Prosseda, G., Bugli, F., & Castagnola, M. (2011). Analysis of heat-induced changes in protein expression of Stenotrophomonas maltophilia K279a reveals a role for GroEL in the host-temperature adaptation. International Journal of Medical Microbiology, 301(4), 273281.*
6. *Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G. O., Azevedo, V.,... & Borriss, R. (1997). The complete genome sequence of the gram-positive bacterium Bacillus subtilis. Nature, 390(6657), 249.*
7. *Kumar, C. S., Mande, S. C., & Mahajan, G. (2015). Multiple chaperonins in bacteria—novel functions and non-canonical behaviors. Cell Stress and Chaperones, 20(4), 555574.*
8. *Maatallah, J., Berraho, E. B., Munoz, S., Sanjuan, J., & Lluch, C. (2002). Phenotypic and molecular characterization of chickpea rhizobia isolated from different areas of Morocco. Journal of applied microbiology, 93(4), 531540.*
9. *Amrein, K. E., Takacs, B., Stieger, M., Molnos, J., Flint, N. A., & Burn, P. (1995). Purification and characterization of recombinant human p50csk protein-tyrosine kinase from an Escherichia coli expression system overproducing the bacterial chaperones GroES and GroEL. Proceedings of the National Academy of Sciences, 92(4), 10481052.*
10. *Yadav, N. S., Rashmi, D., Singh, D., Agarwal, P. K., & Jha, B. (2012). A novel salt-inducible gene SbSI-1 from Salicornia brachiata confers salt and desiccation tolerance in E. coli. Molecular biology reports, 39(2), 19431948.*
11. *Aggarwal, R., Gupta, S., Sharma, S., Banerjee, S., & Singh, P. (2012). Cloning and expression of a small heat and salt tolerant protein (Hsp22) from Chaetomium globosum.*
12. *Guglielmi, G., Mazodier, P., Thompson, C. J., & Davies, J. (1991). A survey of the heat shock response in four Streptomyces species reveals two groEL-like genes and three groEL-like proteins in Streptomyces albus. Journal of bacteriology, 173(22), 73747381.*
13. *Vorderwülbecke, S., Kramer, G., Merz, F., Kurz, T. A., Rauch, T., Zachmann-Brand, B. & Deuerling, E. (2004). Low temperature or GroEL / ES overproduction permits growth of Escherichia coli cells lacking trigger factor and DnaK. FEBS letters, 559(1-3), 181187.*
14. *Kumar, M., Yadav, A. N., Tiwari, R., Prasanna, R., & Saxena, A. K. (2014). Evaluating the diversity of culturable thermotolerant bacteria from four hot springs of India. J Biodivers Biopros Dev, 1(127), 2.*

15. *Selby, K., Lindström, M., Somervuo, P., Heap, J. T., Minton, N. P., & Korkeala, H. (2011). Important role of class I heat shock genes hrcA and dnaK in the heat shock response and the response to pH and NaCl stress of group I Clostridium botulinum strain ATCC 3502. Appl. Environ. Microbiol. 77(9), 28232830.*

16. *Wang Li, Wei-Wei Zhang, Ming-Ming Yang, Yu-Lin Chen (2008): Cloning of the thermostable Cellulase Gene from Newly Isolated Bacillus subtilis and its Expression in Escherichia coli. Mol Biotechnol., 40:195–201.*

17. *Karla L. Ewalt, Joseph P. Hendrick, Walid A. Houry, and F. Ulrich Hartl (1997): In Vivo Observation of Polypeptide Flux through the Bacterial Chaperonin System. Cell, Vol. 90, 491–500.*

18. *Tomoyasu.T et al. 2001 Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the E. coli cytosol. Molecular microbiology 40(2) 397413.*

19. *Schmidt, A., Schiesswohl, M., Völker, U., Hecker, M., & Schumann, W. (1992). Cloning, sequencing, mapping, and transcriptional analysis of the groESL operon from Bacillus subtilis. Journal of bacteriology, 174(12), 3993-3999.*

20. *Kamireddi, Madhavi, Edward Eisenstein, and Prasad Reddy. "Stable Expression and Rapid Purification of Escherichia coli GroEL and GroES Chaperonins." Protein expression and purification 11.1 (1997): 4752.*