ResearchArticle
Volume 10:1, 2021

Molecular Biology

ISSN: 2168-9547 Open Access

Overexpression and Denaturation of Biofilm Forming Autoinducer-2 Kinase from Salmonella Typhi

Princy Vijayababu* Gopinath Samykannu and Sundarabaalaji Narayanan

Structural Biology Laboratory, Department of Bioinformatics, Bharathiar University, Coimbatore, Tamilnadu, India

Abstract

Quorum sensing is a cell-to-cell signaling process that allows bacteria to collectively control gene expression, thereby harmonizing activities that are productive only at a high population density such as biofilm formation and virulence factors production. This process is accomplished through the production, secretion, and detection of small chemical signals called autoinducers. Autoinducer-2 kinase (LsrK) is the kinase protein present in the quorum sensing system. Generally; kinase protein has been implicated in the progression of a wide range of diseases, making these signaling molecules key therapeutic targets for pharmaceutical development. Hence, we engrossed on LsrK; Gene was cloned into pET30b (+) with specific primers and over expressed under control of the T7 promoter using *E. coli* BL21 (DE3) bacterial system with 0.1mM IPTG with LB media at 37°C. Denatured LsrK was obtained after treatment GnHCl. These efficient and optimized methods were supportive for refolding and crystallization of *S. typhi* LsrK.

Keywords: Quorum sensing • LsrK • molecular cloning • Denaturation

Introduction

Salmonella typhi are Gram-negative pathogens that causing the diseases ranging from gastroenteritis and diarrhea to life-threatening systemic syndrome including typhoid fever to human. Typhoid fever remains a global health problem, resulting more than 2, 00,000 annual deaths, mostly children in developing countries. Fortunately, with adequate treatment, most patients recover from the acute phase of typhoid; however, 3–5% of individuals who are infected with *S. typhi* develop a chronic infection in the gall bladder. Furthermore, typhi can most often colonize as biofilm to gallbladder and these abnormalities have risk to develop cancer in gallbladder [1].

Quorum sensing is a cell-to-cell signaling process that enables bacteria to collectively control gene expression, thereby synchronizing activities that are productive only at a high population density such as biofilm formation and virulence factors production. This process is accomplished through the production, secretion, and detection of small chemical signals called autoinducers. In *S. typhi*, AI-2 is synthesized by LuxS and accumulates extracellularly, then it is internalized by the Lsr ABC-type transporter, and the internalized AI-2 is phosphorylated by LsrK kinase. Thus, LsrK enzyme is increasingly being recognized as an excellent target for the development of novel kinase inhibitor. One of the reasons behind small number of kinase protein structures in Protein Data Bank (PDB) is largely due to the insolubility of proteins in aqueous solution [2].

The molecular cloning, Overexpression and Denaturation of Biofilm forming Autoinducer-2 kinase (LsrK) from *Salmonella typhi* were standardized with classical methods and various biophysical factors. These efficient and optimized methods were supportive for refolding and crystallization of *S. typhi* LsrK.

***Address for Correspondence:** Princy Vijayababu, Structural Biology, Labratory, Department of Bioinformatics, Bharathiar University, India;*

Received 05 February, 2021; Accepted 19 February, 2021; Published 26 February, 2021

Materials and Methods

Genomic and plamid DNA isolation

The genomic DNA from *S. typhi* Ty21a was isolated using protocol. The plasmid DNA, cloned in expression vector pET30b (+), was isolated using Alkaline Lysis Method. The concentration and purity of DNA was determined by measuring the absorbance at A260 and A260/280 using a UV spectrophotometer (T80+UV/V is spectro photometer, PG Instruments Ltd, UK).

Gene amplification

The PCR reactions were done with gene specific primer. The PCR components were from New England Biolabs (NEB) and done with MULTIGENE OPTIMAX machine. Primers were designed using NetPrimer.

PCR conditions

PCR conditions were optimized so as to give approximately 98% amplification lsrK with *S. typhi* genomic DNA. The reaction mixture was always kept to 100µl. The volumes of constituents are given below:

5X Phusion polymerse buffer	–	20 µl
dNTPs (10 mM)	–	2.0 µl
Forward Primer (100 pM)	–	0.5 µl
Reverse Primer (100 pM)	–	0.5 µl
Genomic DNA (60 ng)	–	2.7 µl
Phusion Polymerase (1.0 units)	–	1.0 µl
H_2O (to make 100 µl)	–	73.3 µl

PCR was carried out in a thermocycler from MULTIGENE OPTIMAX. Standard PCR conditions used for all the reaction were initial denaturation at 98°C for 2 mins and denaturation at 98°C for 45 seconds, annealing 51°C for 50 seconds, extension at 72°C for 1mins followed by 35 cycles, with a final extension at 72°C for 5 mins. The 0.8% agarose gel electrophoresis was carried out in a horizontal matrix of agarose with 1X TAE buffer [3].

Restriction digestion

The restriction digestion was adopted for digesting the amplicon as well as

pET30b (+) vector. The NdeI and HindIII Restriction digestions were performed in 50µl reaction volume along with its appropriate buffer and incubated at 37°C for 3 hrs. Digested products were analyzed by 0.8% agarose gel electrophoresis [4].

Ligation

Appropriate volume of buffer and 1 U Ligase (T4 DNA Ligase (GeNei)) were mixed along with insert and the backbone vector. The reaction mix was made upto 10 µl with RNase free water and incubated was carried out at 4°C for 16 hrs and directly used for the transformation.1:1 and 1:3 vector to insert DNA was maintained. The optimum concentration of insert was calculated using the formula,

Amount of insert =(Amount of vector (ng)) X (kbp size of insert) X (insert: vector molar ratio)

Transformation and colony screening

Competent cells were thawed on ice and used immediately after thawing. 10 µl of the ligation reaction were added into 100 µl of the competent cells, mixed gently by ticking against the tube and left on ice for 30 mins. The cells were subjected to heat shock of 42°C for exactly 90 seconds and were at incubated on ice to this 900 µl of pre-warmed (37°C). LB medium was added and left at 1h and centrifuged at 5000 rpm for 5 mins, 800 µl supernatant was removed and remaining 200 µl of the suspension was spread on LB agar plates and incubated for 16 hrs at 37°C. After 16 hrs transformed plates were stored at 4°C. Transformed individual colonies were tested for positive clones by colony screening using restriction digestion. Over all molecular cloning for LsrK were illustrated in Figure 1

Overexpression of LsrK

Single colony of transformed pET30b (+)-lsrK in E. coli BL21 (DE3) cells inoculated in to 10ml of LB broth (tryptone -2%, yeast extract-5 % and NaCl-1%) and incubated at 37°C overnight (160rpm). From mother culture 1% was re-inoculated into kanamycin (30µg/ml) supplemented LB broth and incubate 3hrs at 37°C for over expression. pET30b (+)-lsrK cells in *E. coli* BL21(DE3) were grown till the cell density reached A600 of 0.6 and induced with 0.1 - 0.4 mM IPTG in LB broth 37°C for 3,6 and 9 hrs. The cell pellet formed with centrifugation (10,000 rpm for 10 mins) and the supernatant discarded. Finally, the overexpression was analyzed using 12% SDS-PAGE and the cell pellets were stored at -80°C.

Optimization of LsrK denaturation

Lysis buffer were added to the pellet and mix thoroughly and kept it on ice for 30 mins incubation. Cell lysis were done through SONICS sonicate at 1:1 ratio (pulse 1mins on 1 off) for 20 mins with 40 % amplitude. Remove the debris by centrifugation of 10,000 rpm for 10 mins, LsrK denaturation were analyzed by 12% SDS-PAGE. Lysis buffer were optimized by varying pH, detergent, chaotropic agent and incubation time [5].

Protein quantification

Protein quantification assay were found to be difficult, as the lipid membrane and detergents may interfere with the assay. Therefore, UV spectrophotometer methods were used to estimate protein concentration by measuring the absorption of UV rays at 280nm. The absorbance maximum in the near UV (280 nm) was due to the amino acids with aromatic residues such as tyrosine and tryptophan. Determining the protein concentration using UV spectrophotometry at 280 nm was based on following equation

Absorbance (280 nm)=[Protein] (M)xExtinction coefficient (M-1 cm-1)x path length (cm)

The extinction coefficient of LsrK is 89,670 M-1 cm-1

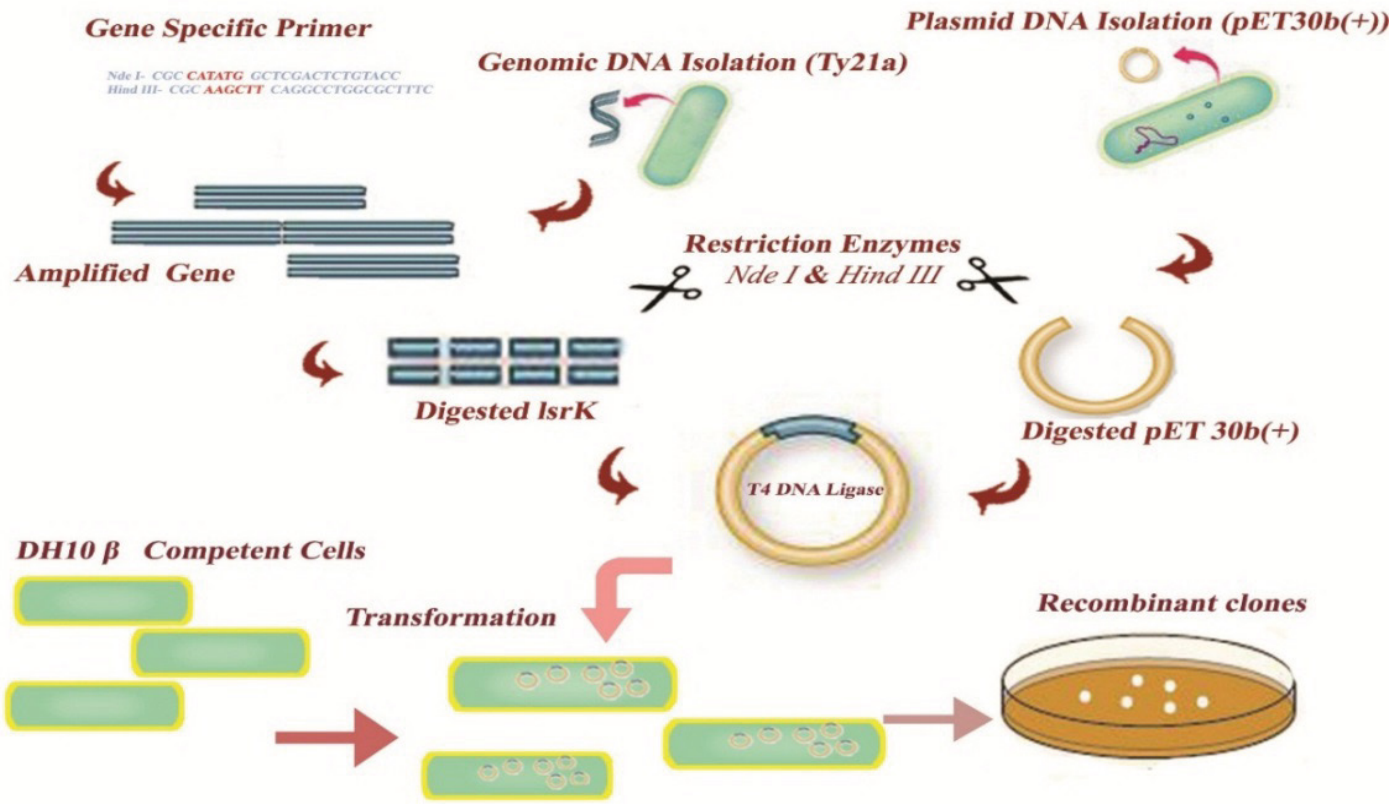

Figure 1. Schematic representation of LsrK molecular cloning. Phusion polymerase is enzyme used to amplify LsrK with specific primers. NdeI and hind III are the enzymes used to restrictions and digestion of LsrK. T4 DNA ligase were used to ligate amplified LsrK and pET30b(+) then the ligated mixture were transformed into *E. coli* DH10βcompetent cells for recombinant LsrK clones.

Results

Genomic DNA and plasmid DNA isolation

The isolated *S. typhi* genomic DNA and plasmid DNA pET30b(+) was found to be pure as verified using 0.8% agarose gel electrophoresis (supplementary Figure 1, Lane 1) and from the absorbance in the visible range of A260/280 ratio. The ratio for the genomic DNA and plasmid DNA fall in the range between 1.8 to 2.0 µg/µl, indicating that they are free from RNA and protein contaminations. The concentration of the DNA and the A260/280 ratio is tabulated in supplementary. Purified genomic and plasmid DNA are used for PCR amplification and restriction digestion analysis [6].

Amplification of LsrK

NdeI (CATATG) and Hind III (AAGCTT) sites were added at 5' and 3' end of the amplicon respectively to enable ligation into pET30b (+) vector. Amplification was done using Phusion DNA polymerase. Amplified lsrK(1593 bp) from genomic DNA was purified from agarose gel electrophoresis and subjected to restriction digestion analysis using appropriate restriction enzymes of NdeI and HindII I(supplementary Figure 2).

Ligation and transformation of lsrK

Amplicon and vector pET30b (+) were digested with restriction enzyme NdeI and Hind III. Digested product of insert and vector was ligated using enzyme T4 DNA ligase. High efficiency competent cells of *E. coli* DH10 cells were transformed with ligation mixture. Finally, recombinant clones pET30b (+) -lsrK were confirmed by restriction digestion analysis and insert was released at the position of ~1593 bp (supplementaryFigure3). **Optimization of LsrK overexpression** Overexpression of LsrK is often very challenging because, each folds in its own unique manner, a process that influenced by the choice of expression host, media, proper induction and time interval (for collecting cell pellet). Thus, no single solution exists for successful production of all recombinant proteins. In this concern, pET30b (+)-lsrK clones were transformed into *E. coli* BL21 (DE3) cells for overexpression. pET30b (+)-lsrK in *E. coli* BL21(DE3) cells were grown; till the cell density was reached at 0.6 of A600 and induced with 0.1mM IPTG in LB broth 37°C for 4, 6 and 16 hrs. The LsrK overexpression was analyze using 12% SDS -PAGE (Figure 2).

Marker

The different concentrations of inducer were used to optimize LsrK overexpression. Once fresh cell density reach to A600 of 0.6 then cultures were induced with 0.1,0.2,0.3 and 0.4 mM IPTG and were allowed to express for 4 hrs at 37°C. Different IPTG concentrations of results presented in supplementary Figure 4. Without lsrK construct were used (*E. coli* BL21 (DE3) pET30b (+)) as control. Supplementary Figure 4 showed that overexpressed LsrK in all IPTG concentrations were produced equally (bandwidth). The lower IPTG concentration (0.1 mM) was enough to induce the LsrK overexpression. These results indicate that a higher IPTG concentration has no significant effect on the LsrK yield. Therefore, further overexpression experiments 0.1 mM IPTG concentration was optimized [7,8].

LsrK- denaturation

Denaturation of kinase protein depends on the intrinsic physiochemical properties. Preparation of the LsrK lysate has critical steps. Optimal conditions maximize cell lysis and the fraction of the recombinant protein is extracted while minimizing protein oxidation, unwanted proteolysis and sample contamination with genomic DNA. Recovering biologically active protein in high yield is become a major problem. In order to accomplish this, LsrK must be solubilized. The common stages involved to recover biologically active and soluble protein include.

Cell disruption

Centrifugation and Washing

Solubilization buffer -tunings

Cell disruption

Whole cell pellet was re-suspended in lysis buffer. Cells were disrupted by ultra-sonic waves generated by SONICS, 130W using an optimized run time of 20 mins with a pulse 3 seconds on and 6 seconds off. Crude inclusion bodies were obtained by centrifugation of the cell lysate at 10,000 g for 10 mins at 4°C [9].

Centrifugation and washing

Crude inclusion bodies were washed four times with Buffer I (50mM Tris-Cl (pH 8.0), 150mM NaCl, 1% Triton X-100, and 1mM PMSF,) and finally washed with buffer II (50mM Tris-Cl (pH 8.0), 10 mM NaCl) once. Re-suspended inclusion bodies were kept at 37°C on incubator shaker for 15 mins. After each wash, the resuspended pellet was centrifuged at 13,000 g for 30 mins at 4°C. Optimization of LsrK lysis buffer components using Lysozyme [10].

Denaturation of LsrK

Denaturation of the purified inclusion bodies were done in various denaturation buffers with different concentrations of Choatropic agents, different temperatures and pH. The optimum buffer composition was found to be 50mM NaH2PO4 pH 8.0, 6M GnHCl, 5mM NaCl, 15mM imidazole and 1mM βMercaptoethanol. Denaturation was done for 12 hrs at 37°C with moderate shaking. Undissolved inclusion bodies were removed by centrifugation at 13,000g for 45 min at 4°C to obtain denaturation LsrK in the Figure 3. The solubilized LsrK was passed through 0.45 µm filter to remove particulate matter.

Molecular weight marker

Denaturation of LsrK from inclusion bodies. Screen with different buffers, pH, different concentration of GnHCL, reducing agent and imidazole were carried out as shown in the Figure 4.

SDS page analysis

After LsrK purification, samples should be resolved by denaturing 12%

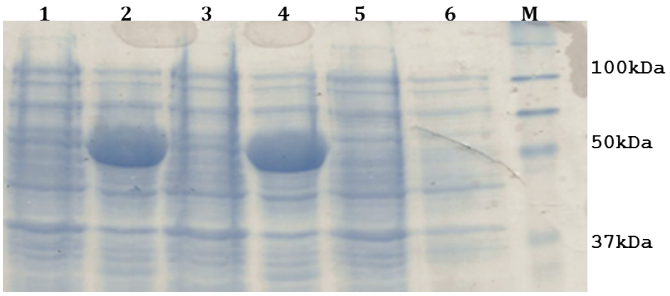

Figure 2. 12% SDS-PAGE analysis of overexpressed LsrK. Lane 1 and 3: A cell free extract of *E.coli* BL21 (DE3) containing pET30b(+)-lsrk Un-induced sample (without IPTG), Lane 2 and 4: A cell free extract of *E.coli* BL21(DE3) containing pET30b(+)-lsrk-Induced sample with IPTG, Lane 5 and 6: A cell free extract of *E.coli* BL21(DE3) containing pET30b (+) induced (with IPTG) and Un-induced sample (without IPTG), Lane M : Protein molecular weight.

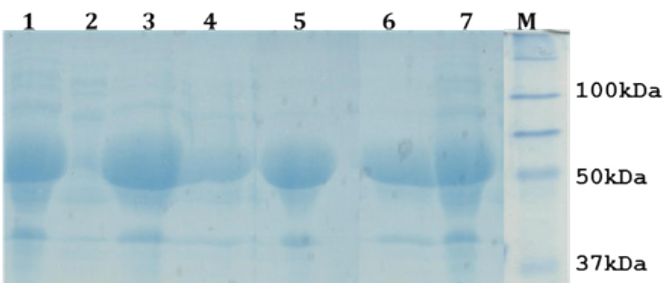

Figure 3. 12% SDS-PAGE analysis of LsrK cell lysis buffer optimization. LsrK whole cell pellet. Lane 2 & 3: Supernatant and pellet of LsrK treated with Tris-Cl (after cell lysis), Lane 4 & 5: Supernatant and pellet of LsrK treated with Potassium phosphate (after cell lysis), Lane 6 & 7: Supernatant and pellet of LsrK treated with Sodium phosphate (after cell lysis), Lane M: Protein.

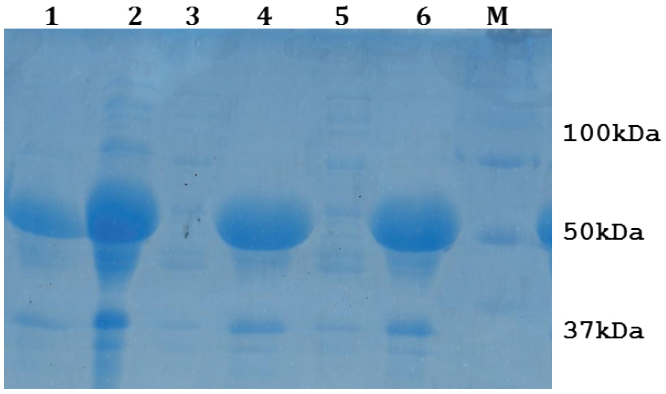

Figure 4. 12% SDS-PAGE showing the optimization of LsrK denaturation. Lane1 & 2 represents: LsrK whole cell pellet & lysate (after sonication), Lane 3 & 4: Supernatant and pellet of LsrK (after centrifugation), Lane 5 & 6: pellet & supernatant and of LsrK denaturation (dissolved in 6M GnHCl), Lane M: Protein molecular weight marker.

SDS-PAGE. If stained with a dye such as Coomassive brilliant blue, the intensity of the bands will usually be proportional to the amount of LsrK. This allows the purity of the sample to be estimated and the expected size denaturation of LsrK was concentrated at 34mg/ml [11].

Discussion

Recombinant cloning and small-scale test expression are extensively used analytical methods in molecular biology. A major concern is the overexpression level and solubility of a recombinant protein may influenced by the culture and other biophysical factors. Generally, physical stability of the protein has a critical concern due to potential for an immune response to protein aggregation. Therefore, solubility and physical stability plays key variables that must be addressed when developing a therapeutic protein. In modern molecular biology, two broad techniques were asses to regulate protein solubility behavior; (1) detection of aggregates and (2) measurement of protein-protein interactions. In this concern, a protein needs to be solubilize before the structure and the mechanism of action. There is no single protocol can be used to separate all types of proteins. Therefore, specific denaturation buffer used depends on the size, Iso electric point, charges and water solubility.

Protein denaturation is the method of breaking interactions elaborate in protein aggregation, which include disulfide bonds, hydrogen bonds, van der Waals forces, ionic interactions, and hydrophobic interactions. If these interactions are not prevented, proteins will aggregate. For effective electrophoretic separation, proteins must be well solubilized. Various agents used for protein denaturation, Cell lysis and protein denaturation are ideally carried out in the buffer that is recommended for the particular electrophoresis technique, especially when native electrophoresis is the method of choice. If this is not possible, proteins must be prepared in sample denaturation solutions that typically contain a number of compounds, including chaotropic agents, detergents, reducing agents, buffers, salts, and ampholytes. In general, a denaturation protocol for protein aggregates, which are critical and must be considered. For the denaturation stage, these variables lie pH, Ionic components of the solvent, Temperature,Time of exposure to solvent, Concentration of the denaturation agent, Concentration of total protein, Presence of reducing agents are very important.

Both pH and ionic strength affect protein solubility; therefore, buffer choice is more important. Many proteins are more soluble at higher pH, using Tris-Cl base is often included to elevate the pH. However, proteins differ in their solubility at different pH values, accordingly the use of different buffers can extract different sets of proteins. The choice of buffer and pH of the sample preparation solution can strongly affect which proteins are effectively solubilized.

Reducing agents such as β-mercaptoethanol (β-ME) and dithiothreitol (DTT) disturb the intramolecular and intermolecular disulfide bonds and used to achieve complete protein denaturation to maintain proteins in their fully reduced states. β-ME is used in large excess in sample buffers to drive the equilibrium reaction towards the completion. If the concentration of β-ME drops and proteins reoxidize, they may yield fuzzy bands upon gel electrophoresis of the sample. Higher concentration of DTT recommended for proteins with large numbers of disulfide bonds.

Chaotropic compounds such as urea and GnHCl disturb hydrogen bonds and hydrophobic interactions within proteins. High concentration of chaotropic agent abolish the secondary protein structure and bring insoluable proteins into solution. Even in the presence of detergents, some proteins have stringent salt requirements to maintain their solubility, but salt should be present only if it is an absolute requirement. If the ionic strength is very high, no visible bands will appear in the lower part of the gel and the dye front will be wavy instead of straight. These are the factors involved in LsrK denaturation.

Conclusion

S. typhi lsrK gene was cloned into pET30b (+) with specific primers and overexpressed under control of the T7 promoter using *E.coli* BL21(DE3) bacterial system with 0.1mM IPTG with LB media at 37°C. The supernatants and pellets obtained after LsrK IBs denaturation using GnHCl suggests LsrK remains in a soluble form as seen on 12% SDS-PAGE and stained with coomassive brilliant blue. We preferred, Guanidine HCl is a stronger chaotropic agent than urea, which can solubilize even extremely sturdy inclusion bodies that are not prone to denaturation by urea. Various strategies have been used for the enhancement of denaturation of inclusion bodies because an efficient denaturation process is necessary for refolding process.

Acknowledgement

Authors thanks to Dr.P. Perumal, for his valuable help in molecular cloning and overexpression.

References

1. Samykannu, Gopinath, Perumal, Perumal. “Computational and Experimental characterization of Integral Membrane Proteins at Molecular Level.” *J Molecul* 8 (2014): 225-258.
2. Kaur, Jasmine and Jain SK. “Vi antigen of Salmonella EnetricaserovarTyphi — Biosynthesis, Regulation and its use as Vaccine Candidate.” *Open Life Sciences* 7(2012): 825-838.
3. Crump, John and Eric Mintz. “Global Trends in Typhoid and Paratyphoid Fever.” *J Clin* 50(2010): 241–246.
4. Crump, John, Stephen P Luby and Eric Mintz. “The Global Burden of Typhoid Fever.” *Bulletin of the World Health Organization* 82(2004): 346–353.
5. Pang, T, MM Levine, B Ivanoff, J Wain and BB Finlay . “Typhoid Fever Important Issues Still Remain.” *Trends in Microbiology* 6(1998): 131–133.
6. Dutta, U, P K Garg, R Kumar and R K Tandon ”Typhoid Carriers among Patients with Gallstones are at Increased Risk for Carcinoma of the Gallbladder.” *Amer J Gastroenterol* 95(2000): 784-787.
7. Lai, CW, RC Chan, AF Cheng and J Y Sung et al. “Common Bile Duct stones: a cause of Chronic Salmonellosis.” *Amer J Gastroenterol* 87 (1992): 1198-1199.
8. Xue, Ting, Liping Zhao, Haipeng Sun and Xianxuan Zhou et al. “LsrR-Binding Site Recognition and Regulatory Characteristics in Escherichia Coli AI-2 Quorum Sensing.” *Cell Research* 19(2009): 1258–1268.
9. Vijayababu, Princy and Samykannu Gopinath. “Structural Insights of cell-cell Communication Autoinducer-2 Kinase (LsrK) model from Salmonella Typhi.” *Int J Current Research* 9 (2017): 51169-51172.
10. Xavier, Karina and Bonnie Bassler. “Interference with AI-2-Mediated Bacterial Cell-Cell Communication.” *Nature* 437(2005): 750–753.
11. Vijayababu, Princy, Gopinath Samykannu and Christian Bharathi. “B-cell and T-cell Epitope Identification with Stability Analysis of AI-2 Import ATP-Binding Cassette LsrA from Typhi in Silico Approach.” *Microbial Pathogenesis* 123(2018): 487–495.

How to cite this article: Vijayababu P, Gopinath S and Narayanan S "Over expression and Denaturation of Biofilm Forming Autoinducer-2 Kinase from Salmonella Typhi." Mol Biol 10 (2021):270.