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Original Article

Expression of Leptospira membrane proteins Signal Peptidase (SP) and Leptospira Endostatin like A (Len A) in BL-21(DE3) is toxic to the host cells

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ABSTRACT

Heterologous expression of Integral Membrane Proteins (IMPs) is reported to be toxic to the host system in many studies. Even though there are reports on various concerns like transformation efficiency, growth properties, protein toxicity, inefficient expression and protein degradation in IMP overexpression, no studies so far addressed these issues in a comprehensive way. In the present study, two transmembrane proteins of the pathogen *Leptospira interrogans*, namely Signal peptidase (SP), and Leptospira Endostatin like A (Len-A) were taken along with a cytosolic protein Hydrolase (HYD) to assess the differences in transformation efficiency, protein toxicity, and protein stability when over expressed in *Escherichia coli* (*E. coli*). Bioinformatics analysis to predict the transmembrane localization indicated that both SP and Len are targeted to the membrane. The three proteins were expressed in full length in the *E. coli* expression strain, BL 21 (DE3). Significant changes were observed for the strains transformed with IMP genes under the parameters analysed such as, the transformation efficiency, survival of colonies on IPTG-plate, culture growth kinetics and protein expression compared to the strain harbouring the cytosolic protein gene.

1. Introduction

Prokaryotic systems for heterologous protein expression are cost effective and easy to manipulate at the genetic level to facilitate expression along with very simple handling options [9]. Their main disadvantage is the lack of sufficient post translational modification machinery to express complex eukaryotic proteins [1,20]. In many cases, over expression of eukaryotic membrane proteins in *E. coli* leads to the accumulation of proteins as inclusion bodies [20,4]. Even though the protein expression procedure, starting from isolation of a target gene is simple and straight forward in principle, the research done by different groups show it as tedious and unrewarding. The difficulties of heterologous protein expression in *E. coli* have been well illustrated [12,30,14].

Among the different strains used for membrane protein overexpression, BL-21DE3 and its derivatives namely C41 (DE3) and C43 (DE3) are the widely used strains. In a study conducted to evaluate the efficiency of different strains to express membrane proteins found that the C41 (DE3) and C43 (DE3) express the proteins (especially the transmembrane proteins) in a better way than that of BL 21 (DE3) [23]. The findings of Wagner and co-workers proved that the expression can be tuned with the mutation in the lacUV5 promoter or by manipulating the polymerase activity and have developed the strain named Lemo21 (DE3) to express the IMPs [34]. Similarly, genetic screening for IMP over expressing strains of *E. coli* resulted in establishing the strain mutant56 (DE3) [36]. In *Bacillus subtilis*, two membrane associated stress responsive system found to be affecting the overexpression of transmembrane proteins and removing the control through mutation improved the yield of heterologous protein [18]. It was shown that when the

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transmembrane protein overexpress, the ratio of membrane lipids and proteins is imbalanced due to the massive insertion of overproduced protein to the membrane which may lead to metabolic stress, formation of inclusion bodies and protein degradation [7,24]. Contradictory to the reports on the use of mutant strains for over expression of IMPs, a study reported that omitting IPTG from the system can perform well and give a continuous expression of IMPs [6].

Among the different strategies used to over express the membrane proteins in *E. coli*, tuning the expression by exploiting the inducer-repressor interaction or induction/post-induction expression temperature, selection of suitable strains for expression and co expression of chaperons are important [32]. Narayanan et al. showed that the operator repressor system used in bacterial expression strains can be fine-tuned to express integral membrane proteins. They have over-expressed a viral ion channel, which is highly toxic to *E. coli* by exploiting the operator repressor interaction [2]. Osterberg and co-workers reported that in Pichia, when the transmembrane protein was over expressed, along with the growth reduction, of the cells, few proteins involved in the stress resistance has been over expressed [25]. Selection of suitable host strain for expression is further depend on the chemical nature of the protein [31].

Massey-Gendel and co-workers used a selection system at the genetic level to screen for mutant strains of fast growing *E. coli* using a C-terminal tagged transmembrane protein. The mutant strains selected when used to express other transmembrane protein also showed good expression [10]. In another approach, random mutations were introduced to eight *E. coli* membrane proteins of different families and analysed the expression of detergent solubilized proteins. It was observed that the expression of five out of nine proteins showed an increase after mutagenesis [8]. Till Gubellini et al. published their work in 2011, there was a common belief that, the expression system and its features are the main reason for the failure of IMP overexpression. The detailed study on the physiological response of the expression strains used in the over expression of heterologous proteins clearly indicate that the normal metabolic process including the biosynthesis of phospholipids, proteins and nucleic acid, aerobic or anaerobic respiration are not hampered seriously. They proposed that the toxicity is attributed to the biochemical and biophysical properties of the over-produced protein, which may facilitate the mutation to improve cell growth [13].

Major objective of the present study was to analyse the effects of IMP over expression on the host cells, mainly the protein toxicity and allied issues like low/no protein expression, growth retardation and difficulty in obtaining the colonies after transformation. Three proteins, two transmembrane proteins and a cytoplasmic protein from Leptospira were selected for expression in *E. coli*. The strain BL-21 (DE3) was used in the study because, most of the negative effects were reported with this strain. The results are explained in line with the possible mechanism reported.

2. Materials and methods

2.1. Materials used in the study

E. coli strain, DH5 alpha was used for cloning and for maintaining plasmids while BL21 (DE3) was used as the expression host. The spirochete *Leptospira interrogans*, serovar Copenhageni strain Fiocruz L1-130 was maintained at the repository of Regional Medical Research Centre (RMRC-ICMR), a WHO collaborating centre for research in Leptospirosis, Port Blair, India. Leptospires were grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium purchased from Difco, USA, supplemented with 1% BSA. The cells were kept at 30 °C with intermittent checking for confluent growth. The fine chemicals, antibiotics, molecular biology kits and reagents were purchased from Sigma, NEB and Merck.

2.2. Selection of target genes and bioinformatics analysis

The two target proteins selected for the study was based on the observation that, the Signal Peptidase (SP) is present in the triton X-114 fraction of Leptospira proteome and Leptospira Endostatin like A (Len A), has been studied earlier [3]. The cytoplasmic protein (HYD) was used as a control. The nucleotide sequences were retrieved from NCBI and confirmed the membrane targeting of the resultant protein using bioinformatics tools. Prediction of secondary structure and positioning of transmembrane spanning regions were done with the deduced amino acid sequences using the programmes available at www.expasy.org/tools; TMpred (http://embnet.vital-it.ch/software/TMPRED_form.html) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). Predictions of solubility was done at http://www.biotech.ou.edu. The sequences obtained after the sequencing of cloned genes in pET28a was compared with the corresponding sequence from NCBI to assure uniformity.

2.3. Cloning of Len, HYD and SP in pET28a

To clone the selected genes for expression, the genomic DNA was isolated from the *Leptospira interrogans*, serovar Copenhageni strain Fiocruz L1-130 using the genomic DNA isolation kit, Himedia. The gene sequences corresponding to the proteins, SP, Hydrolase (HYD) and Len A were amplified from the genomic DNA using the specific primers (HYD-F 5′-AAAGATCTATGAGATCGGAAA GAATTGC-3′, HYD-R 5′-AACTCGAGTTAGATTTGAGAAGAATGCTC-3′, Len A-F 5′-AAAAGCTTGCATGAATTT AAAACAAG G-3′, Len A-R 5′-AACTCGAGTTACTGTTCTACACAGAGAAG-3′, SP-F 5′-AAGGATCCA TGAGTAGAAGTTC TTCCAACC-3′, SP-R 5′- AAGCGGCCGCTTAGAAC GCTTTTCCTAATATG-3′) and cloned in pET-28a (+) expression vector (Novagen-Cat. # 69864-3). The clones were confirmed by sequencing.

2.4. Analysis of transformation efficiency

To analyse the transformation efficiency, the expression constructs (pET28-SP, pET28-Len, and pET28-HYD) were used to transform BL21 (DE3) cells and after one hour incubation at 37 °C, plated on LB agar plates supplemented with kanamycin. The plates were observed for colonies after 14 h. The number of colonies from each of the transformation events done in triplicates were counted manually and the data was used to generate the effect of cloned gene toxicity on transformation.

To analyse the colony survival on IPTG containing plate, the transformed cells were plated on LB agar plates supplemented with Kanamycin (50 μg/ml) and two different concentrations of IPTG (0.4 and 1 mM). The number of colonies were counted after 14 h and the colony morphology was also analysed.

2.5. Protein expression

The protein expression was induced in a 10 ml culture of *E. coli* BL21 (DE3) strain transformed with the constructs pET28-Len, pET28-HYD, pET28-SP. Four IPTG concentrations (0.1 mM, 0.5 mM, 1 mM and 2 mM) and two temperature conditions (37 °C and 25 °C) were analysed for the expression of recombinant proteins. The cells were harvested at every hour after induction by centrifuging 2 ml of the culture at 12,000 rpm for 2 min at 4 °C. The cells were re-suspended in 200 μl of 1X sample buffer and heated in a boiling water bath for 10 min. The sample was

centrifuged at maximum speed for 15 min and the supernatant containing the total protein was analysed using SDS PAGE followed by Coomassie staining.

2.6. Growth kinetics analysis

E. coli BL21 (DE3) cells were transformed with the expression constructs for the growth kinetic studies. *E. coli* BL21 (DE3) transformed with pET28a vector was used as control. Single colony of all the tests and control were inoculated in 2 ml LB media and grown for overnight at 37 °C. The optical density (OD) at 600 nm of overnight grown cultures adjusted to 1.1% was used to inoculate two sets of 100 ml LB and incubated at 37 °C. One set was used to measure the growth for uninduced condition and another for the induced condition. Protein induction was carried out according to the procedure described above using standardized IPTG concentration. Samples of 1 ml were drawn at every 30 min and the OD was measured at 600 nm using spectrophotometer. For the uninduced samples, the media was collected from the time the culture OD reached 0.6. The experiments were conducted in triplicate to confirm the reproducibility. For each sample the mean and standard error were calculated using statistical functions from Microsoft Excel.

3. Results

3.1. Identification of the target genes

Among the peptides identified in the proteome analysis of the triton fraction of *Leptospira interrogans* (Data not published), two proteins (SP and Len) were selected randomly for the study. The nucleotide and corresponding amino acid sequences were retrieved from NCBI for the genes selected. Analysing the membrane spanning regions of the proteins in the study using the software's TMpred as well as TMHMM revealed that, the proteins have transmembrane domains in them with a prediction score of 2100 for SP (residues 41–61) and 1700 for Len (residues 55–73). The transmembrane domain in both SP and Len dominated by the hydrophobic residues such as, Valine, Leucine, Isoleucine and phenyl alanine. The amino acid sequence of HYD did not show any transmembrane domains in the analysis. While both SP and Len showed 0% solubility, the Hyd was shown to be 100% soluble when expressed in *E. coli*.

3.2. Generation of expression constructs

The gene sequences of Len, HYD and SP were amplified using the specific primers and cloned in the expression vector pET28a. The restriction enzyme combination *Bam HI-Xho I* (Len and HYD) and *Bam HI-Not I* (SP) were used to confirm the presence of DNA fragments representing the full length genes in pET28a vector.

3.3. Transformation of BL21 (DE3) with the expression constructs

To examine the effect of protein expression on host cells, the expression constructs pET28-HYD, pET28-Len and pET28-SP along with pET28a plasmid were used to transform the expression strain BL21 (DE3). When the number of colonies obtained were analysed, there was nearly 60% reduction in terms of colony count in both pET28-Len and pET28-SP compared to pET28-HYD. The number of colonies observed with pET28-HYD was matching with the number of colonies obtained in the transformation event using the pET28a vector alone (Fig. 1). Among the constructs of SP and Len, the cells transformed with SP showed less number of colonies. No significant difference was observed among the sizes of colonies

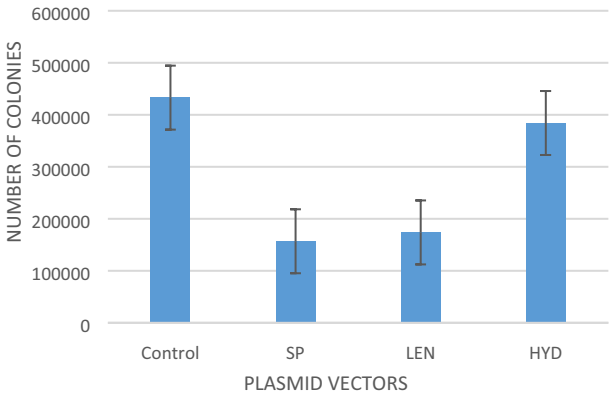

Fig. 1. Transformation efficiency of Len, SP and HYD. The BL21 (DE3) cells transformed with the plasmids, pET28a (control), pET28-SP, pET28-Len and pET28-HYD and plated on the LB-agar plates with the antibiotic Kanamycin (50ug/ml). The colonies developed were counted and the transformation efficiency was calculated. The experiment repeated thrice to ensure the accuracy.

obtained in the transformation events. When the cells were plated on IPTG plates, there was a further reduction in the number of colonies in pET28-Len and pET28-SP, but the colony number did not show any significant variation for pET28-HYD under both IPTG and non-IPTG conditions (Fig. 2). The signal peptidase showed lesser number of surviving colonies than Len. The results were matching with the transformation efficiency experiments in BL21 (DE3) cells.

3.4. Protein expression and analysis

The expression pattern of the candidate genes showed maximum expression at defined combinations of IPTG concentration and post induction temperature. When the expression was checked after 30 min, very feeble expression has been observed in Len and SP. Even though HYD showed protein expression at 30 min, the samples from 1 h only were used in the analysis to ensure uniformity. While the expression at 37 °C did not show any difference among the membrane proteins and the control (HYD), at 25 °C, the membrane proteins showed the expression pattern slightly higher than HYD (Fig. 3). The IPTG concentration was crucial in SP as the low concentration tried was favouring the expression than 1 or 2 mM of IPTG. In the case of Len, high concentration of IPTG increased the expression compared to the low concentration. In HYD expression, not much difference was observed between the different concentrations of IPTG used. The sizes of the proteins

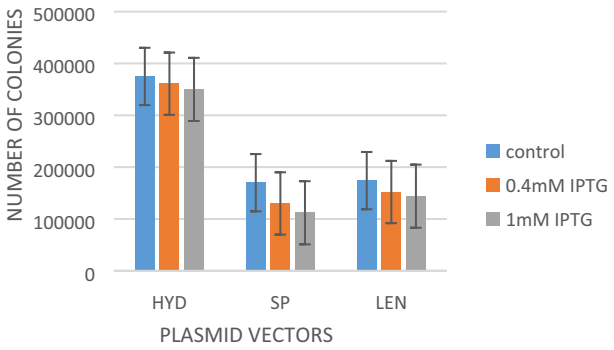

Fig. 2. On plate screening of colonies for protein expression. The BL21 (DE3) cells transformed with the plasmids, pET28-SP, pET28-Len and pET28-HYD were plated on the LB-agar with the antibiotic Kanamycin (50 μg/ml) and IPTG (0.4 mM and 1 mM). The colonies developed were counted and plotted against different IPTG concentrations. The experiment repeated thrice to ensure the accuracy.

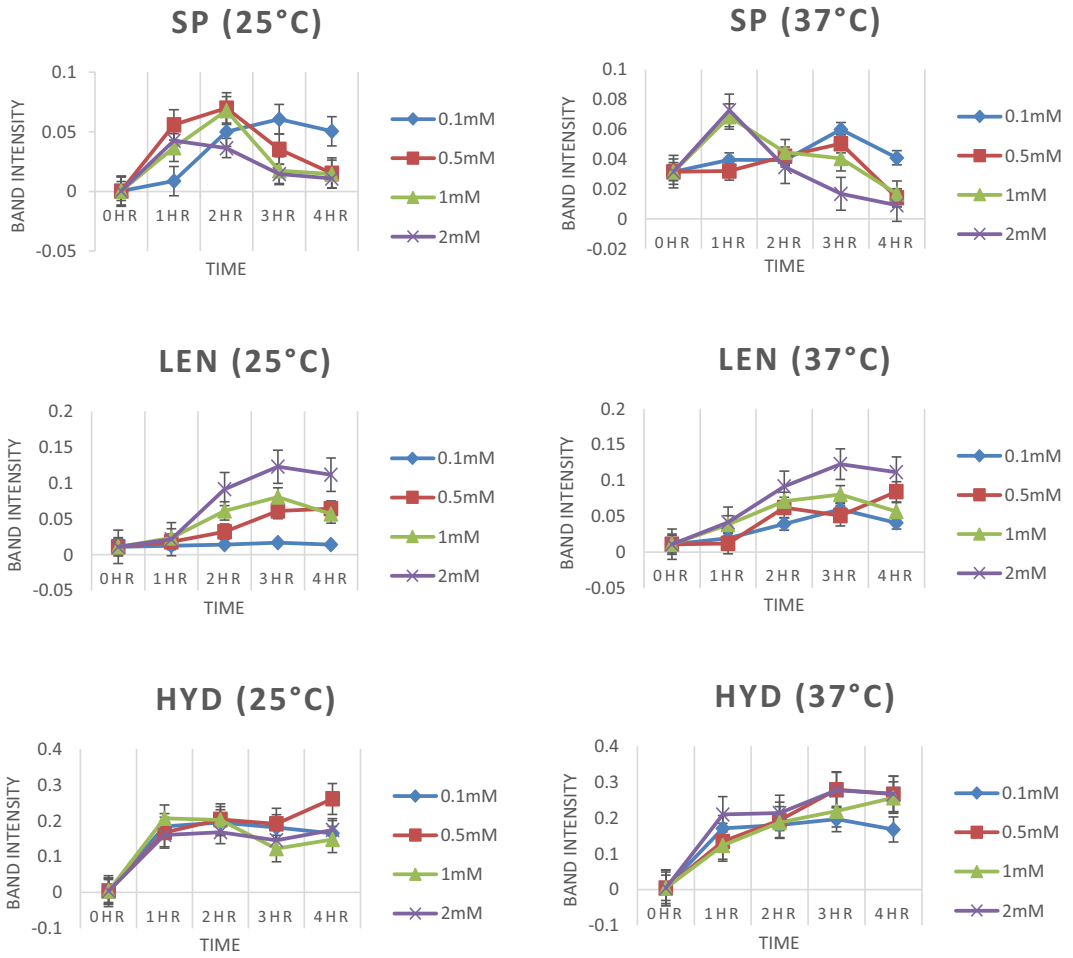

Fig. 3. Expression of Len, SP and HYD under 25 °C and 37 °C. The protein expression constructs pET28-SP, pET28-Len and pET28-HYD were used to transform BL 21 (DE3) cells and the proteins were expressed *in vitro*. The protein expression was induced with different concentrations of IPTG (0.1, 0.5, 1, and 2 mM) and incubated at two (25 °C and 37 °C) post induction temperatures. Samples were collected at different time intervals and analysed through SDS-Page followed by Coomassie staining. The band intensity of the expressed protein on the SDS-PAGE was used to generate the data to create the graphs.

estimated according to the protein molecular weight marker from the SDS-PAGE was 21, 26 and 30 kDa for SP, Len and HYD respectively. The molecular weights were matching with the molecular weight of the full length proteins predicted through bioinformatics tools. As the protein expression was less with the membrane proteins, the experiments to find out the quantity of protein expressed in soluble and insoluble fractions were not performed. Coomassie stained gels for the proteins are given (Fig. 4).

3.5. Growth retardation analysis

In order to study the effect of integral membrane protein expression in heterologous system, the growth retardation of BL-21 (DE3) transformed with the expression constructs was analysed spectrophotometrically after inducing the protein expression using IPTG. While the uninduced culture followed a steady increase in cell density, the induced culture of BL 21 (DE3) transformed with both Len and SP showed a decline in cell density to more than 50% compared to the control (Fig. 5). Induction of HYD expression did not seem to inhibit the cell growth and multiplication.

4. Discussion

Membrane proteome analysis of the pathogen, *Leptospira interrogans* revealed the presence of a significant number of proteases

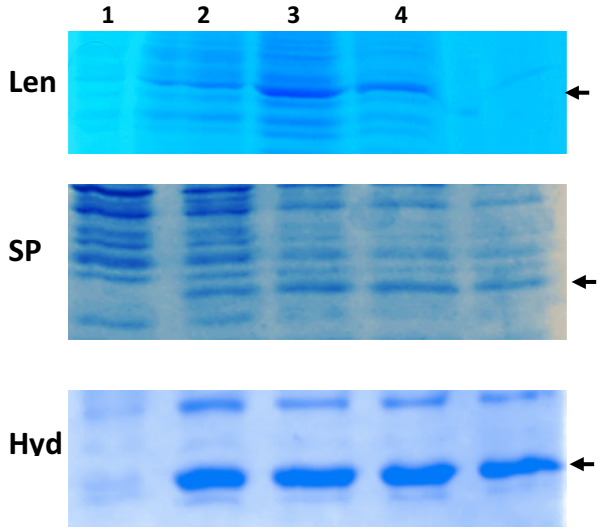

Fig. 4. Coomassie stained SDS PAGE of Len, SP and HYD. The culture of BL21 (DE3) cells containing different expression constructs were induced with 0.5 mM IPTG and kept at 25 °C for protein expression. Samples were collected at different time intervals (1 h, 2 h, 3 h, and 4 h) and the total protein was prepared. The samples were analysed on a 12% SDS PAGE. Lane 1- Uninduced, lane 2, 3, 4 and 5- samples collected at 1, 2, 3 and 4 h respectively. Arrow indicate the expressed protein band.

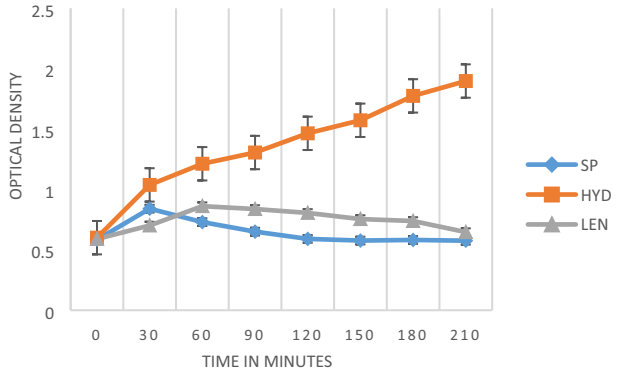

Fig. 5. Growth analysis of BL 21 (DE3) harbouring pET28-HYD, pET28-Len and pET28-SP. The BL21 (DE3) cells transformed with the expression constructs pET28-HYD, pET28-Len and pET28-SP were induced for protein expression at OD, 0.6 and the cell density was monitored after every 30 min spectrophotometrically for a period of 4 h.

along with the membrane proteins. While the selected genes HYD and SP have not been expressed in heterologous systems, cloning and expression of Len A was reported earlier [3,5]. Heterologous expression of IMPs reported to be negatively affecting the host system and the indications of the same are explained in terms of protein toxicity, low/no expression of IMPs, decline of cell growth etc. [11,20]. Over the years, different groups have reported the development of strains of *E. coli*, which are capable of overexpressing IMPs [23,36]. In the study, the strain BL21 (DE3) was selected over strains BL21 (DE3) pLys, C41 (DE3) or C43 (DE3) to analyse the reported negative effects in different publications, which can occur in a standard expression strain.

Difficulties in transforming the expression strains with the expression construct harbouring the IMP genes have been reported in microbes [4,21,23,26,27,33]. The manifestations include low number of colonies after transformation, smaller size of the colonies under the IPTG selection pressure and mutations which can occur in the host genome [16,17]. In our experiments, compared to the vector alone control and pET28-HYD, the IMP gene constructs, pET28-Len and pET28-SP produced nearly 60% less colonies after transformation (Fig. 1). The cytoplasmic protein construct pET-HYD showed almost equal number of colonies as that of the vector alone control.

On-plate protein induction using IPTG while plating followed by transformation or adding the IPTG while pre-culturing before plating are the indirect strategies to assess the toxicity of cloned protein and to screen for mutants resisting the toxic effects of overexpressed IMPs [29,30]. When IPTG was introduced on plates, the Len and SP containing colonies showed a decline in number compared to the control pET-HYD. The pattern observed for both Len and SP were same as that of the results obtained for transformation efficiency (Fig. 2). The results indicate that there may not be a direct effect of the expressed protein on the survival of the host as there was no significant difference in the transformed colony number under both IPTG and non-IPTG conditions. The differences which can be expected due to the vector and copy number toxicity (at the transcript level) [6] has been avoided by using the same vector in the cloning for all the experiments.

The influence of IPTG on protein induction found to be confirming the reports that low concentration of IPTG supports heterologous IMP expression [35,37]. Negative effects of IPTG when interacting with certain gene products was reported as the reason for low expression of cloned genes [16]. In the IPTG plate experiments, a higher concentration of IPTG reduced the colony number in both Len and SP. For the protein expression studies, four different IPTG concentrations were used at two different temperature. The results did not give a significant correlation to the IPTG concentration, protein expression and temperature. In general, while a low temperature (25 °C) regime was found to be better than 37 °C (Fig. 3), no such observations were made for IPTG concentration. It may be concluded that the IMP expression is highly variable and unpredictable as in many cases [4,5,32]. The observations are also supported with the reports that (i) in BL 21 (DE3) IMP overexpression was observed in overnight culture without the addition of IPTG [19,38] and (ii) omitting the IPTG helped in a steady expression of IMP without affecting the normal metabolism of the host [38]. So the results indicate the necessity of careful standardizations in terms of inducer concentration when using BL 21 (DE3) as the expression system.

Difference in growth kinetics of expression strains used for the heterologous expression of many proteins including IMPs and toxic proteins have been reported. The causative factors including the toxicity of expressed proteins, metabolic burden due to the overconsumption of precursors, and toxicity due to the interaction of inducer with cellular components control the cell growth and culture density [7,11,15,21,22,28]. In many of these reports, the protein expression level was directly correlated with the decline in cell growth. In the present study, while the control protein (pET-HYD) did not show any variation in growth pattern with a steady increase in the cell density, the pET-Len and pET-SP cultures showed a decline in growth rate in a time dependent manner (Fig. 5).

5. Conclusions

The study demonstrates the expression of two membrane proteins (Len and SP) and a cytoplasmic protein (HYD) of Leptospira in BL21 (DE3). The sizes of the proteins expressed was matching with the predicted sizes of the proteins based on the deduced amino acid sequences from the gene. The different variables assessed to analyse the toxicity of expressed proteins showed that the expression of IMPs may cause toxicity and growth retardation. Even though the on-plate induction experiments indicate that the expressed protein may not be toxic to the system, the targeting or the overexpression is somehow harming the normal metabolism of the host. Detailed analysis are needed to examine whether the amino acids in the membrane spanning regions have any role to play with these toxic effects.

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Conflict of interest

The authors declare no conflict of interest.

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