Construction of New Genetic Tools for Protein Overexpression in *E. coli-Pseudomonas* host systems

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Abstract

This study reported the construction of a new Escherichia coli-Pseudomonas shuttle vector for overexpression of elastase strain K in both E. coli and Pseudomonas as well as for rapid purification using the new RTX-tag. A 6.5 kb novel shuttle vector, designated as pSIT/RTX, was constructed from pCon2(3) in order to improvise the expression of pCon2(3). pSIT/RTX was harbour a tightly regulated promoter $P_{T7(A1/O4/O3),}$ for controlling gene expression; stabilising fragment (SF) for replication and the maintenance of plasmid in E. coli and P. aeruginosa; attB gene for genome integration; elastase strain K as the passenger enzyme and RTX-tag rapid purification. E. coli TOP10/pSIT/RTX was chosen to proceed with purification as the highest amount of proteolytic activity was detected at 12 h after incorporation with 0.6 mM IPTG (Isopropyl β- d-1-thiogalactopyranoside); the pSIT/RTX showed a clear difference between culture in Luria-Bertani broth without ampicillin (5.4 $X10^3$ CFU) and 3.6 $X10^3$ CFU for culture in ampicillin.

***Keywords**: pSIT*/RTX; overexpression; elastase strain K; rapid purification; RTX-tag

Introduction

Recently, various techniques have been developed for over-expressing and purifying the desired recombinant protein in a bulk quantity due to the enormous potential for biotechnology application [1]. Yet, the progress of technique development is still continuously being made due to some problems that are often found throughout the process including but not limited to protein inactivity, inclusion body (IB) formation, poor growth of the host, and even the failure to achieve any protein at all [2]

In 2017, Wong and colleagues reported that the constructed vector known as pCon2(3), which harboured two expression cassettes was unable to express significant protease activity in *E. coli*. However, in *Pseudomonas aeruginosa*, PAO1, pCon2(3) was able to overexpress greater elastinolytic activity. This is the consequence from the fact that pCon2(3)/*E. coli* TOP10 is capable to utilise *lac* promoter for protein expression only.

Therefore, the improvised shuttle expression vector is designed in this study to achieve a higher expression of protease in both *E. coli* and *Pseudomonas* strains. The new construct of pSIT/RTX will be greatly assisted by the modified T7 promoter/repressor, $T7_{(A1/O4/O3)}$. The fact that the tightly regulated T7 promoter system consists of two lac promoter sites- *O3* and *O4*, makes it efficient in controlling the expression level ([3] and important to avoid leaky expression [4]. Lower controlling of the promoter by RNA polymerase exhibits a higher rate of promoter clearance, which in turn increases the repression factor [4-7].

Literature Review

The primary goal of gene vector construction is to tune vector expression to the maximum level of protein production while minimising the input cost and time. Nonetheless, in many circumstances, a lesser protein production rather than a maximum production is desirable. At a certain point, proteins could become harmful and toxic to the growth of the cell which indirectly make protein ineffectively folded and unable to express at high levels. However, a rapid development in the molecular biology field has now made vector possible to be overexpressed at desired points.

Presently, the most common expression system, *Escherichia coli* has been used in recombinant protein production since it is a well-characterised genome with a variety of mature tools which are obtainable for genetic manipulation [8]. Unfortunately, genes from *Pseudomonas aeruginosa* and other *Pseudomonas* hosts cannot be expressed at an adequate level in *E. coli* [9]. The ability for a cloning vector to be replicated in both *E. coli* and *Pseudomonas* is contributed by two different origins of replication [10] which are *rep* (pMB1) in *E. coli* and the stabilising fragment (SF) for *Pseudomonas,* respectively [11]. Therefore, in this research, the stabilising fragment (SF) from the pUCP19 multiple cloning sites (MCS) was used

Besides SF, an insertion of a strong promoter can optimise the yield of gene expression [12]). Considering that, a well-studied promoter that was suitable for a high level of protein expression in *E. coli* was selected for this research. $T7_{(A1/O4/O3)}$ consists of *A1* which is known as a stronger promoter compared with other commonly used promoter including *E. coli* operons P_{lac}, P_{tac} and P_{bla} [3]. [13] constructed a suite of promoter by inserting *lacI* operator sequence into two sites; the first site was a sequence that consisted of 29 bp native *E. coli* lac operon in position homologous to P_{lac} *(O3)* and the other one was *O4*, which was a sequence that carried 17 bp core region of the wild type lac operator as a spacer between -10 and 133 hexamers. In consequence of the specific interaction of the operator or promoter region and the DNA polymerase, the spacing between -10 and -35 is generally tightly conserved [14]. Comprehensively, the $T7_{(A1/O4/O3)}$ promoter contains

a dual *lac* operator sites for the binding of the Lac repressor that is expressed by *lacI*^{q}, which makes it able to be repressed tightly. ([3, 10, 13, 15-20]

According to [21], gene fusion technology offers an analysing expression of many genes that are encoded by bacterial genome to maintain the plasmid in a particular host. In order to enhance the integration of vector in the *Pseudomonas*, a certain gene is needed, for example, the specialised mini-CTX vectors containing the insertion of the gene cassette of 30 bp *attB* sequence that have been integrated in the *P. aeruginosa* host strains. This permits the expression from *T7* and *lac* promoters and is useful for studying gene expression using *lac* and *lux*-based reporter genes [22].

Nowadays, the scientist is eager to over-express their protein in order to facilitate the next steps in research like purification, crystallisation and protein structure. Therefore, in this research, in order to enhance rapid purification, repeat-in-toxin (RTX-tagged) has been added to the construct; the RTX-tagged belongs to the Family 1.3 lipase. This family has the ability to secrete lipases by accommodating three components of ATP-binding cassette (ABC) transporter system (one step pathway) [23, 24]. Protein that is secreted under the ABC transporter has several repeats of nine-residue GGXGXDXUX sequence motif (X: amino acid; U: hydrophobic residue) at C-terminal secretion signal [25]. The first six residues of this motif form a loop and each Ca^{2+} ion binds between pairs of the loop. This repetition forms a β-roll structure motif [25].

Due to these characteristics, a construction of genetic and molecular tools consists of: (1) 1.8 kb *Pst*I stabilising fragment (SF) (ATCC 87110) for replication and maintenance of plasmid in both *E. coli* and *P. aeruginosa*, (2) a tightly regulated $T7_{(A1/O4/O3)}$ promoter or repressor system for control of gene expression [26], (3) the 30 bp *attB* sequence for integration of gene cassettes into *Pseudomonas* genome [27], and (4) RTX -tag for purification purpose was carried out.

Methods/Methodology

Source of bacteria and plasmids

Cultures of *E. coli* DH5a and TOP10 which are harbouring pUCP19 (ATCC 87100) and pTEL are readily available in the laboratory. Respectively, the *E. coli* strain- TOP10 (Invitrogen, USA), BL21 (DE3) (Invitrogen, USA) and KRX (Promega, USA), was used as competent cells; while the culture media LB broth (Difco, USA) was supplemented with ampicillin at the 100 µg/mL as final concentration were routinely used.

Construction of vectors

Shuttle vector, pSIT/RTX, harbours a stabilising fragment (SF) for the replication and maintenance of plasmid in *E. coli* and *P. aeruginosa*, *T7(A1/O4/O3)* promoter for controlling gene expression, RTX-tag for binding calcium for a purpose of rapid purification, *attB* gene for integration genome in PAO1, β-lactamase gene for antibiotic resistance and Origin which is the region of the plasmid for replication. Figure 1 represents an overall flow work of the vector construction. Concurrently, a cassette carrying the elastase strain K and *T7(A1/O4/O3)* of pTEL (Shamsudin et al., 2017) was removed in order to be replaced with a synthesised gene that consisted of the RTX motif tag, a tightly regulated *T7(A1/O4/O3)* promoter and an elastase in order to give rise to pLIP. Finally, the constructed pLIP was digested with *Ssp*I and ligated to a 30 bp *attB* sequence to give rise to pSIT/RTX. The used of 1 % agarose gel electrophoresis was applicable for analysing all DNA fragments including the constructs and was purified by the Gel Extraction Kit (Qiagen, USA) as directed by the manufacturer. Normal standard protocol

was used for Bacterial transformation of pSIT/RTX into *E. coli* TOP10, BL21(DE3) and KRX as well as *Pseudomonas aeruginosa* PAO1. Screening of proteolytic activity of the transformed bacteria were done by using skim milk agar that was supplemented with ampicillin (100 µg/mL). The formation of a clearance zone around the colonies exhibited successful test results.

Analysis of pSIT/RTX from *E. coli* TOP1

White colonies harbouring the recombinant plasmids were isolated, QIAprepMiniprep Kit (QIAGEN, Germany) were used to purify and visualise according to the manufacturer's instructions. The purified sample was visualised on 1.0 % (w/v) agarose gel. Analysis of the plasmids by restriction enzyme digestion (*Pst*I and *Hind*III) and PCR method with M13 Forward primer (-20): 5' GTAAAACGGCCAG 3' and M12 Reverse primer: 5' CAGGAACAGCTATGAC 3', were carried out to confirm the presence of insert and PCR products, respectively.

DNA sequencing of pSIT/RTX

Purified recombinant plasmid was sequenced using M13 primers. Analysis of the sequence, primary structure and database similarity was performed by using Basic Local Alignment Search Tool [28], Expasy Molecular Biology Server [16] and Biology Workbench ([29].

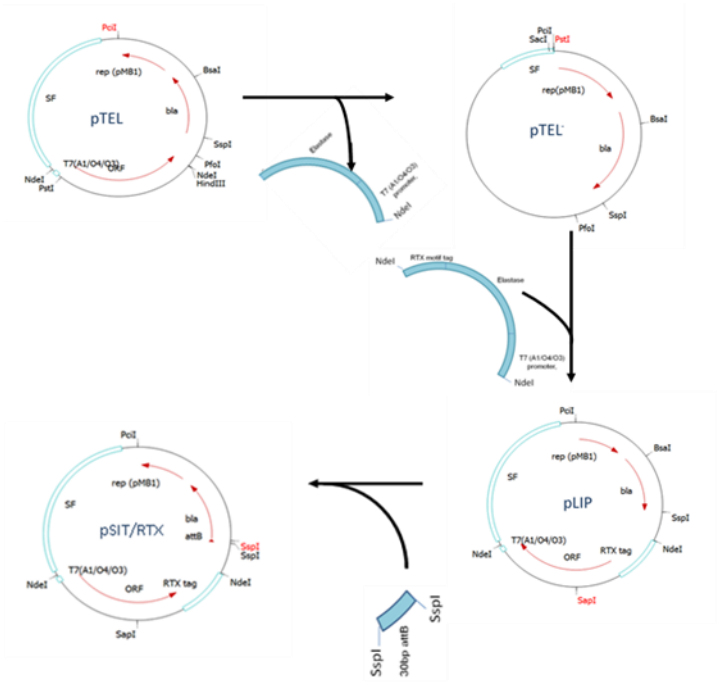

Figure 1 Construction of Recombinant Plasmid; pSIT/RTX. (Diagram is drawn using Clone Manager and does not accordingly to scale)

Proteolytic Assay

Azocasein (0.5 %, 1 mL) as a substrate was mixed with an enzyme and incubated at 37 °C. After 10 minutes, 20 % TCA was added to stop the reaction. The mixture had been vortexed before it was incubated at a room temperature for 5 minutes. In order to get supernatant, the mixture was then centrifuged at 10, 000 rpm for 5 minutes at 4 °C. Then, it had been added with 1 mL of 1 M NaOH for every 500µL of supernatant. Proteolytic activity was observed using A_{440nm}. The recombinant plasmid in each strain of both the *E. coli* and *P. aeruginosa* was done in triplicate where the empty plasmid acted as a control. The proteolytic assay was followed by the [30] methodology with some modification.

Optimization of Protein Expression

The constructed pSIT/RTX of *E. coli*/TOP10 was selected for further optimisation study due to its highest proteolytic expression that was exhibited during the pre-screening experiment. For optimisation of induction time, the culture was harvested at every 4 hours after induction- ranging from 0-43 hours. After evaluation of the induction time, the analysation of an optimum concentration of IPTG (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) was tested. Then, *E. coli* TOP10/pSIT/RTX was induced at different A_{600nm} ranging from 0-1 at 0.25 intervals.

Results and Discussion

Construction of pSIT/RTX

The removal of pTEL fragment by a single digestion using *Nde*I was confirmed by the presence of a 2.5 kb band of pTEL on the agarose gel (Figure 2). The pLIP was electrophoresed on agarose gel to confirm that the plasmid consisted of pTEL and $T7_{(A1/O4/O3)}$ promoter, elastase strain K and RTX-tag. The existence of constructed plasmid, pLIP with the size of 3.0 kb from recombinant *E. coli* TOP10 was visualised in Figure 3 (A). Continuing from that, pLIP was digested with restriction endonuclease, *Nde*I. *Nde*I was digested pLIP into two fragments due to the presence of *Nde*I linker in the interest fragment prior to ligation to pTEL. Figure 3 (B) illustrates the release of the interest fragment (2.2 kb) which harbours $P_{T7(A1/O4/O3)}$, the ORF of organic solvent tolerant elastase strain from *P. aeruginosa* strain K and MCS of pUC18 from pLIP. Therefore, pLIP was deduced to encode 6.4 kb of nucleotides.

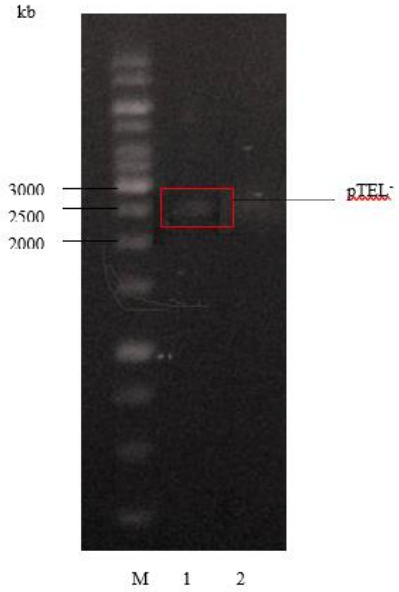

Figure 2 Construction of pTEL-. Lane M: GeneRuler™ 1 kb DNA Ladder (Fermentas, USA); Lane 1 and 2: pTEL-.

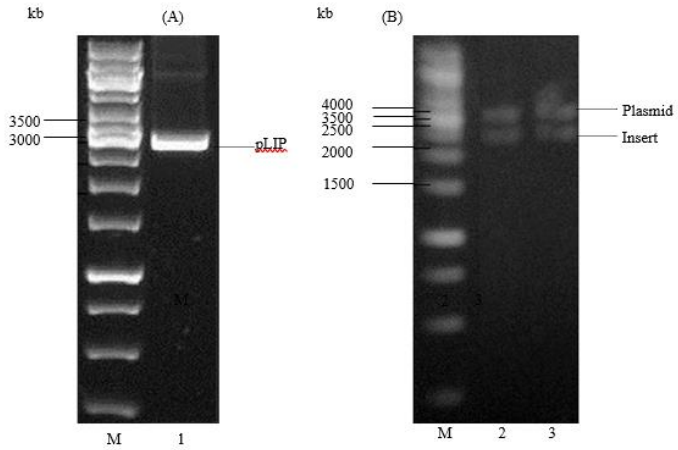

Figure 3 Gel electrophoresis from the constructed plasmid pLIP. (A) Extraction of pLIP from *E. coli* TOP10/pLIP. (B) Single digestion of pLIP by *Nde*I. Lane M: GeneRuler™ 1 kb DNA Ladder (Fermentas, USA); Lane 1: pLIP;Lane 2 and 3: *Nde*I digested-pLIP.

A 900 kb band appeared as the result of the singe digestion [Figure 4 (A)]. PCR did not work for confirmation of *attB* ligation since 30 bp is too small to be visualised from the 1 % gel. However, PCR was replaced with the transformation of pSIT/RTX into the *Pseudomonas* strain PAO1. The integration of pSIT/RTX into *Pseudomonas* genome showed the success of *attB* ligation with pLIP. From the plasmid extraction analysis [Figure 4 (B)], there is no band appearance as the construct has been successfully integrated with PAO1.

To double confirm the presence of 30 bp *attB* and PCR products, purified recombinant plasmid were sent for DNA sequencing to 1st Base Laboratories Sdn. Bhd. (Malaysia). The physical map is displayed in Figure 5.

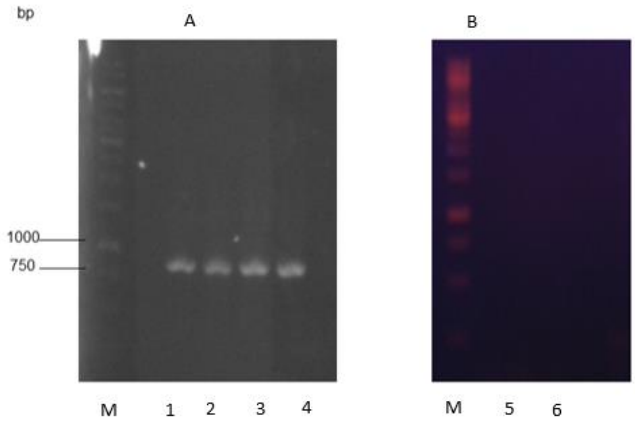

Figure 4 Confirmation of insertion 30 bp *attB* gene (A) Confirmation of 30 bp *attB via* single digestion. (B) Conformation of genome integration in *P. aeruginosa* PAO1. Lane M: GeneRuler^{TM} 1 kb DNA Ladder (Fermentas, USA); Lane 1, 2, 3 and 4: *Ssp*I-digested-pSIT/RTX; Lane 5 and 6: genome integration.

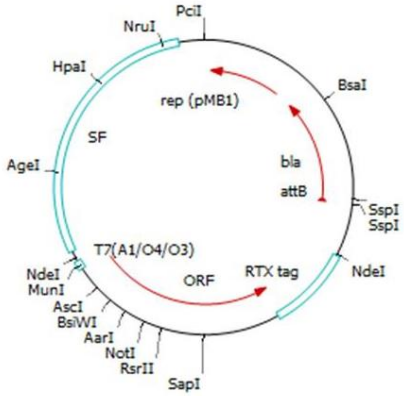

Figure 5 Physical maps of constructed shuttle vectors with an expression cassette controlled by $P_{T7(A1/O4/O3)}$, pSIT/RTX. Maps are generated by Clone Manager 9 Basic Edition (Scientific and Educational Software, USA) and not drawn according to scale.

Expression Analysis of pTEL and pSIT/RTX on Various Strains of *E. coli* and *Pseudomonas aeruginosa* Strain PAO1

The optimum proteolytic activity was recorded as shown in Figure 6. TOP10/pTEL and TOP10/pSIT/RTX showed the highest proteolytic activities, i.e. 127.56 and 98.76 U/mL. BL21(DE3)/pTEL showed the lowest proteolytic activity (83.00 U/mL) while for pSIT/RTX, the lowest proteolytic activity was in KRX (91.90 U/mL). However, small differences of proteolytic activity were recorded between BL21(DE3) and KRX as each construct was slightly small. Nevertheless, when all *E. coli* strains were compared to the *Pseudomonas aeruginosa* strain PAO1, the strain PAO1 showed the highest proteolytic activity for pSIT/RTX, i.e. 157.67 U/mL. On the other hand, for pTEL, the proteolytic activity recorded was 98.76 U/mL.

The *E. coli* TOP10 is incapable to utilise the $T7_{(A1/O4/O3)}$ promoter for protein expression [26]. This study has reported that both TOP10/pTEL and TOP10/pSIT/RTX have shown the highest proteolytic activity which are 127.56 and 98.76 U/mL, respectively. [27] has indicated that isopropyl β-D-thiogalactoside (IPTG) is not required to induce expression

from *lac* promoter in *E. coli* TOP 10. Therefore, [27] statement could be the reason why high level of expression was observed after an induction with 1.0 mM IPTG in this research. A comparative analysis across a panel of *E. coli* strains including TOP10, BL21(DE3) and KRX demonstrated that TOP10 was the most suitable strain for recombinant enzyme overexpression.

In contrast, the lowest proteolytic activity in BL21(DE3)/pTEL was partly contributed by the accumulation of acetate and the presence of elastase in the insoluble state. For the first reason, the outcome of growth an *E. coli* BL21(DE3) in a media containing glucose was accumulation of acetate [31]. Luria-bertani agar and broth containing a certain amount of glucose is described by Lennox (Difco™ & BBL™). [10] stated incubation of *E. coli* BL21(DE3) at high growth rate (A_{600nm}=1.8) resulted in accumulation of negative by-product which is acetate. The accumulation of acetate was encouraging the decrease of protein production.

E. coli KRX also facing a same problem with *E. coli* BL21(DE3) which is elastase was in insoluble state. However, [32]found an additional reason which led to the low expression of recombinant DNZR, i.e. the suitability of the tag with the host. The uses of low-strength promoter and auto-induction in *E. coli* KRX can overcome the toxicity problem and enhance the high output of protein [20]).

Meanwhile, the recombinant plasmid pTEL and pSIT/RTX had exhibited proteolytic activities of 94.56 and 157.67 U/mL, respectively. This showed that the signal and propeptide in the structural gene of the elastase strain K were recognized by the inner and outer membrane translocation machinery of PAO1 as a host. The dissociation of signal peptide in the inner membrane occurred following the translocation of proenzyme across the outer membrane. According to [17], the proenzyme was degraded by an extracellular protease to acquire active mature elastase.

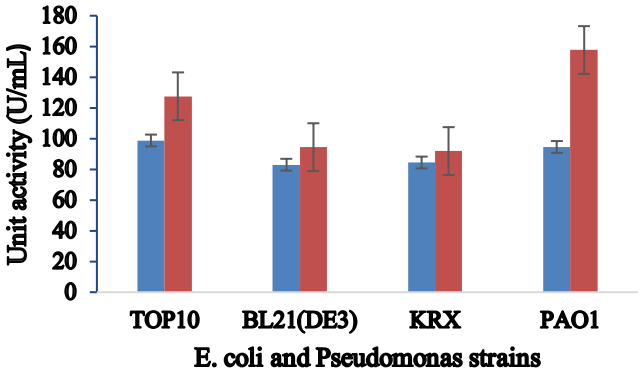

Figure 6 Expression of organic solvent tolerant elastase strain K of pTEL(■) and pSIT/RTX (□) in various type of *E. coli* and *Pseudomonas* strains. The cell lysate was analysed for it proteolytic activity in various type of *E. coli* strain at 6 hr induction time of 1.0 mM IPTG at A_{600nm}= 0.5. Activities are represented by mean value ± standard deviations (n= 3).

Optimization of Elastase Strain K

A parametric study was conducted on *E. coli* TOP10/pSIT/RTX prior to the highest expression exhibited during pre-screening (Figure 6). The purpose of this parametric study is to optimise the induction conditions in the recovery of the elastase strain K enzyme from the overexpression of *E. coli* TOP10/pSIT/RTX. The induction of IPTG promotes the expression of the elastase which in turn prevents the promoter from being repressed.

Despite this, *lac* promoter is not metabolised by the *lac* operon product and does not require continuously in the medium as the cell grow. Table 1 shows that 4 h of induction is sufficient to generate large amounts of active protein. Therefore, without the need of overnight induction, protein can be expressed and analysed in less time. At 12 h after induction, the optimum proteolytic activity expression was detected with the OD reading of 186.89 U/mL. Induction time of 4h and 8 h faced a gradually increased from 119.11 to 176.56 U/mL, respectively. On the other hand, the proteolytic activity was decreased progressively starting from 16 to 24 h with the observation of 172.00, 122.67 and 95.33 U/mL, respectively. This was support by [11]where the plasmid recombinant would exhibit overexpression if the induction time was longer.

The effect of IPTG on the efficiency of the induction has been examined using different IPTG concentrations. IPTG was used as inducer as it cannot be metabolised by the organism [33]. Hence, its concentration remains constant during the entire experiment. IPTG has the ability to induce lac-repressor-regulated promoters to enhance the expression of protein [34]. Several concentrations of IPTG were used to obtain optimum protein yields. Lower concentration of IPTG will led to insufficient yield while higher concentration of IPT cause toxicity to the cell [19].

Table 1 shows proteolytic activity has been observed at all tested IPTG concentration at optimum level of 192.33 U/mL after 12 h of induction by 0.6 mM IPTG. Under the similar condition, IPTG concentrations of 0.2 and 0.4 mM showed a significantly high proteolytic activity of 116.44 and 133.33 U/mL, respectively. After additional IPTG concentration to 0.8 and 1.0 mM, a gradual decrease of proteolytic activity of 153.22 and 111.22 U/mL were produced respectively.

According to [35], a lower concentration of inducer caused inefficient induction, a result of low recombinant protein yields. On the other hand, a higher inducer concentration has driven the toxicity effects such as decrease in cell growth and recombinant protein concentration [36]. A study that was done by [30] showed that by culturing at a lower temperature, a selection of different *E. coli* strains, the substitution of selected amino acid residues, the co-production of chaperon and modification of the culture medium's pH affected the production of soluble protein.

The differences in lag time or growth rate typically cause a situation where various cultures are ready for induction at different times [9]. For the study of correlation between cellular growth at the time of induction and elastase strain K production, cultures were induced at the different growth phases. Table 1 clearly shows the optimum induction at the late exponential phase which has produced a high level of proteolytic activity (294.44 U/mL). The resulted induction of A_{600nm} at 0.25, 0.50 and 0.75 showed a gradual increased from 126.11 to 175.56 and finally 237.89 U/mL, respectively. The increasing of proteolytic activity that had happened due to the IPTG induction at high density resulted in the function of soluble proteins and a high efficiency of fractionation [37].

Furthermore, a non-significant proteolytic expression level of 14.89 U/mL was observed at A_{600nm} of 0.00 as the IPTG induction at low cell density resulted in the less specific productivity per cell [19, 24]. [18]showed that at the early log phase, protein expression could not be detected However, rapid and high-level production was monitored as the growth rate approaching saturation.

Induction at the stationary phase (A_{600nm}=1.2) exhibits the decreasing of protein expression level [4]. A pro-longed 15 h incubation was devoted to saturate culture density of overgrowth cells that had lost plasmid, thus, resulted in reduction of protein expression [38].

Table 1

Optimization of Elastase Strain K

Effect	Interval	Protease Activity (U/mL)	Standard deviation
Induction time (h)	0	17.667	0.027
	4	119.111	0.056
	8	176.556	0.034
	12	186.889	0.022
	16	172.000	0.067
	20	122.667	0.045
	24	95.333	0.033
IPTG concentration (mM)	0.0	14.778	0.008
	0.2	116.444	0.017
	0.4	133.333	0.008
	0.6	192.333	0.039
	0.8	153.231	0.062
	1.0	111.232	0.025
Induction optical density at 600nm	0.00	14.889	0.003
	0.25	126.111	0.013
	0.50	175.556	0.019
	0.75	237.889	0.049
	1.00	294.444	0.023
	1.25	237.444	0.022

Conclusions

The pSIT/RTX offer a new alternative shuttle expression vector for protein overexpression in *E. coli* and *Pseudomonas*. The existence of dual repression genetic elements in pSIT/RTX, the *O3* and *O4*, and also the tightly regulated P_{T7} promoter has further repressed basal expression in tested bacterial strain and thus has countered all the detrimental effects that are caused by the basal expression, as discussed earlier. Furthermore, the $P_{T7(A1/O4/O3)}$ has driven the overexpression of active protein, as shown by the high proteolytic activities under optimised conditions. Our recent modification of pSIT/RTX which is accommodated with *attB* gene has shown that this construct can be replicated independently with a high copy number and is incorporated stably into the genome of *Pseudomonas*. This demonstrate that the SF region's role for plasmid maintenance is not only applicable in *Pseudomonas* but is also extended to its related genus which includes *Burkholderia* sp. and *Klebsiella* sp.

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References

1. Assenberg, R., et al., *Advances in recombinant protein expression for use in pharmaceutical research.* Current opinion in structural biology, 2013. **23**(3): p. 393-402.DOI: https://doi.org/10.1016/j.sbi.2013.03.008.
2. Baneyx, F. and M. Mujacic, *Recombinant protein folding and misfolding in Escherichia coli.* Nature biotechnology, 2004. **22**(11): p. 1399-1408.DOI: https://doi.org/10.1038/nbt1029.
3. Harris, A.J., *Development and application of variable strength expression vectors in Shewanella oneidensis MR-1*. 2014.
4. Osasumwen, O.F., et al., *Implications of the release of Chibok girls on Nigeria's war on terrorism.* Covenant University Journal of Politics and International Affairs, 2017. **5**(1).
5. Coêlho, D.F., et al., *Azocasein substrate for determination of proteolytic activity: Reexamining a traditional method using bromelain samples.* BioMed Research International, 39-62., 2016. **2016**.DOI: https://doi.org/10.1155/2016/8409183.
6. De Mey, M., et al., *Minimizing acetate formation in E. coli fermentations.* Journal of Industrial Microbiology and Biotechnology, 2007. **34**(11): p. 689-700.DOI: https://doi.org/10.1007/s10295-007-0244-2.
7. Dehkordi, A.J. and H.M.M. Sadeghi, *Optimization of Vasopressin Type 2 Receptor Expression in Escherichia coli BL21*. 2016.
8. Liu, L., et al., *How to achieve high-level expression of microbial enzymes: strategies and perspectives.* Bioengineered, 2013. **4**(4): p. 212-223.DOI: https://doi.org/10.4161/bioe.24761.
9. Rolfe, M.D., et al., *Lag phase is a distinct growth phase that prepares bacteria for exponential growth and involves transient metal accumulation.* Journal of bacteriology, 2012. **194**(3): p. 686-701.DOI: https://doi.org/10.1128/JB.06112-11.
10. Maj, A., et al., *Plasmids of carotenoid-producing Paracoccus spp.(Alphaproteobacteria)-structure, diversity and evolution.* PLoS One, 2013. **8**(11): p. e80258.DOI: https://doi.org/10.1371/journal.pone.0080258.
11. Wong, C.F., et al., *Organic-solvent stability of elastase strain K overexpressed in an Escherichia–Pseudomonas expression system.* Biotechnology and applied biochemistry, 2010. **57**(1): p. 1-7.DOI: https://doi.org/10.1042/BA20100224.
12. Pitzer, J., et al., *Novel DNA and RNA elements*, in *Synthetic biology*. 2016, Springer. p. 65-99.DOI: https://doi.org/10.1007/978-3-319-22708-5_2.
13. Lanzer, M. and H. Bujard, *Promoters largely determine the efficiency of repressor action.* Proceedings of the National Academy of Sciences, 1988. **85**(23): p. 8973-8977.DOI: https://doi.org/10.1073/pnas.85.23.8973.
14. Fulcrand, G., et al., *DNA supercoiling, a critical signal regulating the basal expression of the lac operon in Escherichia coli.* Scientific reports, 2016. **6**(1): p. 1-12.DOI: https://doi.org/10.1038/srep19243.
15. Egharevba, O., *Cloning and Expression of Four Laccase genes isolated from Arctic marine Psychrobacter strains in Escherichia coli and psychrophilic Pseudomonas.* 2017.
16. Gasteiger, E., et al., *ExPASy: the proteomics server for in-depth protein knowledge and analysis.* Nucleic acids research, 2003. **31**(13): p. 3784-3788.DOI: https://doi.org/10.1093/nar/gkg563.
17. Hoge, R., et al., *Weapons of a pathogen: proteases and their role in virulence of Pseudomonas aeruginosa*. Current research, technology and education topics in applied microbiology and microbial biotechnology, 2010. **2**: p. 383-395.
18. Madar, D., et al., *Promoter activity dynamics in the lag phase of Escherichia coli.* BMC systems biology, 2013. **7**(1): p. 1-13.DOI: https://doi.org/10.1186/1752-0509-7-136.
19. Márquez, D.L. and L.M. García, *Evaluation of Plasmid Stability by Negative Selection in Gram-negative Bacteria.* Bio-protocol, 2017. **7**(9).DOI: https://doi.org/10.21769/BioProtoc.2261.
20. Moore, S.J., et al., *EcoFlex: a multifunctional MoClo kit for E. coli synthetic biology*. ACS Synthetic Biology, 2016. **5**(10): p. 1059-1069.DOI: https://doi.org/10.1021/acssynbio.6b00031.
21. Zhang, J., et al., *INTEGRATE: gene fusion discovery using whole genome and transcriptome data.* Genome research, 2016. **26**(1): p. 108-118.DOI: https://doi.org/10.1101/gr.186114.114.
22. Gilbertsen, A. and B. Williams, *Development of a Pseudomonas aeruginosa agmatine biosensor.* Biosensors, 2014. **4**(4): p. 387-402.DOI: https://doi.org/10.3390/bios4040387.

23. Chenal, A., A.C. Sotomayor Perez, and D. Ladant, *Structure and function of RTX toxins.* The comprehensive sourcebook of bacterial protein toxins, 2015. **4**: p. 677-718.DOI: https://doi.org/10.1016/B978-0-12-800188-2.00023-9.
24. Morgan, J.L.W., J.F. Acheson, and J. Zimmer, *Structure of a type-1 secretion system ABC transporter.* Structure, 2017. **25**(3): p. 522-529.DOI: https://doi.org/10.1016/j.str.2017.01.010.
25. Sotomayor-Pérez, A.-C., D. Ladant, and A. Chenal, *Disorder-to-order transition in the CyaA toxin RTX domain: implications for toxin secretion.* Toxins, 2015. **7**(1): p. 1-20.DOI: https://doi.org/10.3390/toxins7010001.
26. Wong, C.F., et al., *Construction of New Genetic Tools as Alternatives for Protein Overexpression in Escherichia coli and Pseudomonas aeruginosa.* Iranian Journal of Biotechnology, 2017. **15**(3): p. 194.DOI: https://doi.org/10.15171/ijb.1524.
27. Yamabhai, M., et al., *Efficient E. coli expression systems for the production of recombinant β-mannanases and other bacterial extracellular enzymes.* Bioengineered bugs, 2011. **2**(1): p. 45-49.DOI: https://doi.org/10.4161/bbug.2.1.13419.
28. Altschul, S.F., et al., *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research, 25(17): 3389-3402.* . 1997.DOI: https://doi.org/10.1093/nar/25.17.3389.
29. Subramaniam, S., *The Biology Workbench--a seamless database and analysis environment for the biologist.* 1998. p. 1-2.
30. Choi, T.-J. and T.T. Geletu, *High level expression and purification of recombinant flounder growth hormone in E. coli.* Journal of Genetic Engineering and Biotechnology, 2018. **16**(2): p. 347-355.DOI: https://doi.org/10.1016/j.jgeb.2018.03.006.
31. Wang, H., et al., *Improving the expression of recombinant proteins in E. coli BL21 (DE3) under acetate stress: an alkaline pH shift approach.* PloS one, 2014. **9**(11): p. e112777.DOI: https://doi.org/10.1016/j.pep.2014.01.008.
32. Schröder, C., et al., *Identification and expression of genes involved in the conversion of daidzein and genistein by the equol-forming bacterium Slackia isoflavoniconvertens.* Applied and environmental microbiology, 2013. **79**(11): p. 3494-3502.DOI: https://doi.org/10.1128/AEM.03693-12.
33. Rizkia, P.R., et al., *Effect of Isopropyl-β-D-thiogalactopyranoside concentration on prethrombin-2 recombinan gene expression in Escherichia coli ER2566.* Procedia Chemistry, 2015. **17**: p. 118-124.DOI: https://doi.org/10.1016/j.proche.2015.12.121.
34. Ravishankar, S., et al., *An IPTG inducible conditional expression system for mycobacteria.* PLoS One, 2015. **10**(8): p. e0134562.DOI: https://doi.org/10.1371/journal.pone.0134562.
35. Dolan, K., et al., *Global burden of HIV, viral hepatitis, and tuberculosis in prisoners and detainees.* The Lancet, 2016. **388**(10049): p. 1089-1102.DOI: https://doi.org/10.1016/S0140-6736(16)30466-4.
36. Shafiee, F., M. Rabbani, and A. Jahanian-Najafabadi, *Optimization of the expression of DT386-BR2 fusion protein in Escherichia coli using response surface methodology.* Advanced biomedical research, 2017. **6**.DOI: https://doi.org/10.4103/2277-9175.201334.
37. Malik, A., et al., *Optimization of expression and purification of HSPA6 protein from Camelus dromedarius in E. coli.* Saudi Journal of Biological Sciences, 2016. **23**(3): p. 410-419.DOI: https://doi.org/10.1016/j.sjbs.2015.04.017.
38. Alfasi, S.N., *Physiological aspects underpinning recombinant protein production in Escherichia coli.* 2011.