

Construction and Evaluation of Self-Directing Expression System Using Regulatory Elements of Cry Gene of *Bacillus Thuringiensis*

Drishtant Singh (✉ drishtantsingh@yahoo.com)

Guru Nanak Dev University <https://orcid.org/0000-0003-0179-2057>

Samiksha .

Guru Nanak Dev University

Seema Madhumal Thayil

Guru Nanak Dev University

Satwinder Kaur Sohal

Guru Nanak Dev University

Anup Kumar Kesavan

Guru Nanak Dev University <https://orcid.org/0000-0002-0156-0769>

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Abstract

An expression system based on the *cry* gene regulatory elements was constructed. The Terminator region of *cry* gene from *B. thuringiensis subsp. kurstaki HD-1* was cloned in pSG1151 plasmid downstream to gfp_{mut1} . The promoter region of the *cry* gene was amplified to give three different reading frames. The Promoter region of *cry* gene was cloned in pSG1151T plasmid upstream to gfp_{mut1} . The expression of GFP under the promoter/terminator expression system was evaluated by checking the expression of gfp_{mut1} under the same promoter. The GFP content of pSG1151 and three constructs; pDSA1, pDSA2 and pDSA3 were compared by fluorescence spectroscopy. The fluorescent intensity of pSG1151 and pDSA1 were compared at time interval of 6 hours upto 72 hours. Both the samples showed detectable fluorescence that increased with time up to 12 hours, but the increase in the fluorescence of pDSA1 was 3 times higher as compared to pSG1151. A cold peptidase gene was cloned under the control of the *cry* promoter. The transformed *E.coli* DH5 α colonies were patched on skim milk agar plates and the clones of pSG1151CP and pDSA1CP were compared on the basis of zone of clearance. The zone of clearance of pDSA1CP was much higher as compared to that of pSG1151CP. The cell-free supernatant of *Bacillus sp. S1DI 10* and recombinant pDSA1CP collected at different time points was assayed for the specific activity of the extracellular protease. At 72 hours the protease activity in pDSA1CP was 2.7 fold higher compared to that of wild *Bacillus sp. S1DI 10*.

1. Introduction

The accumulation of Cry toxin protein inside the bacillary body leads to the formation of crystal inclusions in *B. thuringiensis*. The possible mechanism behind the accumulation of crystal protein by *B. thuringiensis* is the expression of crystal protein gene via a strong promoter. The promoter is active during both in logarithmic and in stationary phase resulting in the overproduction of the crystal protein. The strains of *Bacillus sp.* respond to nutritional deficient conditions by either becoming; dormant or by sporulation. On this basis, the *cry* genes that are expressed in the stationary phase can be divided into two categories; *cry* genes contingent on spore formation and *cry* genes that are not related to spore formation (Agaisse and Lereclus, 1995).

The endospore formation in *Bacillus sp.* takes place in a sporangium which consists of two cellular compartments called as the forespore and the mother cell. The process of endospore development is regulated by a primary sigma factor of vegetative cell and five other sigma factors that appear successively during sporulation. The recognition of gene promoters selectively depends upon the binding of these sigma factors to RNA Polymerase (Helmann et al., 1988, Moran, 1993). The Cry toxin secreted by *cryIA* gene is an example of *cry* gene expressed in mother cell of *B. thuringiensis* and is associated with sporulation. In addition to sigma factors, two overlapping promoters (BtI and BtII) for *cryIA* were mapped by Wong et al., (1983) which is active during different time points in sporulation phase. The initiation of transcription by the RNA Polymerase from these two promoters carries separate sigma factors (Calogero et al., 1989, Brown and Whiteley, 1990). The different sigma factor mutants of *cryIA-lacZ* fusion in *B. thuringiensis* gave either diminished or no β -galactosidase activity. However, a very high β -galactosidase

activity was observed in wild *cryIA-lacZ* fusion of *B. thuringiensis*, which suggests that strong promoters are involved in the expression of *cryIA* gene (Bravo et al., 1996). Various studies have shown that some *cry* genes in different strains of *B. thuringiensis* carry only Btl or both the promoters and others have regions similar to those in Btl and BtII promoters (Brown and Whiteley, 1988, Brown, 1993, Dervyn et al., 1995, Hajime et al., 1993). Thus, these *cry* genes can be categorized as sporulation dependent *cry* genes.

The *cryIIIa* gene is an example of sporulation independent *cry* genes as its expression was independent of the sigma factors present during sporulation in both *B. thuringiensis* and *B. subtilis* *cry* gene (Agaisse and Lereclus, 1994a, Salamitou et al., 1996). The expression of *cryIIIa* was increased in mutant strains of *B. thuringiensis* which were not able to commence sporulation. (Lereclus et al., 1995). Smith, (1993) identified two different regulators in stationary phase which are required for gene expression in *B. subtilis*.

Further, the location of a gene on plasmid affects its expression level as the copy number of plasmid has been exploited in recent times for overexpression of a protein. The *cry* genes are also carried by plasmids which naturally lead to a huge quantity of toxins in various *B. thuringiensis* strains. The presence of different *cry* genes varies in different strains of *B. thuringiensis* which differs in size and shape of the crystal (Lereclus et al., 1993). However, the cloning of *cryIaC* gene in a strain having other *cryI* genes lead to lower production of Cry protein as compared to its cloning in *cry⁻* strain while there was no decline in its expression when cloned into strains with *cryIIIa* gene (Baum et al., 1990, Lecadet et al., 1992, Lereclus et al., 1992). Therefore, the expression of *cry* gene may not be related to the high copy number of plasmid.

mRNA has a specific half life and its degradation affects the expression of a gene. The increase in expression of a protein requires production of a stable mRNA as evident from *ompA* mRNA that encodes a membrane protein in *Escherichia coli* having longer half life of 20 minutes as compared to 2-3 minutes for the other mRNAs in *E. coli* (Nilsson et al., 1984). The mRNAs that encode crystal protein in *B. thuringiensis* have a half life of ~10 minutes (Glatron et al., 1972). In addition to that, the 3' terminal region of the *cryIAa* gene acts as a positive retro-regulator in *B. thuringiensis* as its heterologous expression with a gene increased the mRNAs half life as well as the expression (Wong and Chang, 1986, Wong et al., 1983). The terminal region consists of inverted repeats forming stem loop structure that prevents degradation of mRNA from exonucleases like exoribonuclease PNPase in *E. coli* (Causton et al., 1994). An ~600bp upstream promoter containing two distinct regions is involved in expression of the *cryIIIa* gene in *B. thuringiensis* (De Souza et al., 1993, Agaisse and Lereclus, 1994b). The upstream region was reported to be involved in transcription while the downstream region acts as a 5' mRNA stabilizer which increased the stability of mRNA as well as the expression of protein. (Bechhoffer et al., 1993, Hue et al., 1995, Agaisse and Lereclus, 1996).

Therefore, the present study was undertaken to construct promoter/terminator based expression system harboring promoter and terminator region of *cry* gene from *B. thuringiensis subsp. kurstaki* HD-1.

2. Materials And Methods

2.1 Plasmid and strains collection

The reference strains *B. thuringiensis subsp. kurstaki* HD-1 (BGSC Accession no. 4D1), plasmid vector pSG1151 containing *gfp_{mut1}* (BGSC Accession no. ECE 152) and a *cry⁻* *B. thuringiensis subsp. kurstaki* HD CRY-1 (BGSC Accession no. 4D7) were kindly provided by Dr. Daniel R. Zeigler (Director of the BGSC).

2.2 Isolation of genomic DNA and plasmid DNA

The reference strain *B. thuringiensis subsp. kurstaki* HD-1 was grown overnight for the isolation of genomic DNA according to the method described in a previous study with slight modifications (Valicente *et al.*, 2008, Singh *et al.*, 2019). The strains carrying plasmid (pSG1151) were grown overnight for isolation of plasmid DNA using High Speed Plasmid Mini Kit (IBI, Scientific, USA) following the manufacturer's instructions.

2.3 PCR amplification of terminator region of *cry* gene

The DNA isolated from *B. thuringiensis subsp. kurstaki* HD-1 was used as template for the amplification of promoter and terminator region of the *cry* gene. The primers for the terminator region of *cry* gene (TFP1 5'-GCTCTAGACGTGGACA GCGTGG AATTA-3' and TRP2 5'-TCCCCGCGGTAAGTTGCTCTATACATA-3') were designed on the basis of the sequence determined in a previous study (Wong and Chang, 1986). The terminator region was amplified in a 20 μ L reaction mixture containing Phusion High Fidelity buffer (5X), 10 μ M each of forward and reverse primer, 10 mM dNTPs, 20 ng/ μ L DNA, and 2U Phusion DNA Polymerase (NEB, USA). The PCR reaction was performed in a Thermocycler (Agilent Technologies, USA) with an initial denaturation step of 98°C for 30 sec, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 20 seconds. A final step of extension was performed at 72°C for 10 min. The PCR-amplified product was purified using a PCR/Gel Purification Kit (IBI, Scientific, USA) following the manufacturer's instructions.

2.4 Cloning of terminator region of *cry* gene

The PCR amplified product was purified and digested with restriction enzymes; XbaI and SacII (NEB, USA) at 37°C for 4 hours. pSG1151 plasmid was digested overnight with the aforementioned restriction enzymes at 37°C. The digested PCR product and vector were ligated using T4 DNA Ligase (NEB, USA) at 16°C for 16 hours and transformed into *DH5a* strain of *E. coli*. The transformed cells were plated on ampicillin (100 μ g/ml) supplemented LB Agar plates. After incubation at 37°C for 18 hours, clones with successful recombination showing ampicillin resistance were selected and multiplied.

2.5 PCR amplification of promoter region of *cry* gene:

The primers for the promoter region of *cry* gene (Table 1) were designed from a previous study that determined nucleotide sequence of promoter region of *cry* gene (Wong *et al.*, 1983). The promoter region was amplified in a 20 μ L reaction mixture containing Phusion High Fidelity buffer (5X), 10 μ M each of forward and reverse primer, 10 mM dNTPs, 20 ng/ μ L DNA, and 2U Phusion DNA Polymerase (NEB, USA). The PCR reaction was performed in a Thermocycler (Agilent Technologies, USA) with an initial

denaturation step of 98°C for 30 sec, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. A final step of extension was performed at 72°C for 10 min. The PCR-amplified product was purified using a PCR/Gel Purification Kit (IBI, Scientific, USA) following the manufacturer's instructions.

Table 1
Primers for the promoter region of *cry* gene

S. No.	Name	Sequence
1.	PFP1	5'- GGGGTACC TGTATAAGTGTAAGTAAG -3'
2.	PRP1	5'- CCGCTCGAG CATAAGTTACCTCCATCT-3'
3.	PRP2	5'- CCGCTCGAG CCATAAGTTACCTCCATCT-3'
4.	PRP3	5'- CCGCTCGAG TCCATAAGTTACCTCCATCT-3'
5.	UVPRP1	5'- CCCAAGCTT CATAAGTTACCTCCATCT-3'
6.	UVPRP2	5'- CCCAAGCTT CCATAAGTTACCTCCATCT-3'
7.	UVPRP3	5'- CCCAAGCTT TCCATAAGTTACCTCCATCT-3'

2.6 Cloning of promoter region of *cry* gene:

The PCR amplified product was purified and digested with restriction enzymes; KpnI and XhoI (NEB, USA) at 37°C for 4 hours for the construction of pDSA1, pDSA2 and pDSA3. pSG1151T (this study) was digested overnight with the aforementioned restriction enzymes at 37°C. The digested PCR product and vector were ligated using T4 DNA Ligase (NEB, USA) at 16°C for 16 hours and transformed into *DH5a* strain of *Escherichia coli*. The transformed cells were plated on ampicillin (100 µg/ml) supplemented LB Agar plates. After incubation at 37°C for 18 hours, clones with successful recombination showing ampicillin resistance were selected and multiplied.

2.7 GFP expression analysis by fluorescence spectroscopy:

pSG1151 plasmid carrying strain along with the three strains carrying the vectors constructed in this study were grown in LB broth at 37°C for 48 hours at 180 rpm in an orbital shaker (Scigenics, India). The cells were harvested with GFP extraction buffer (25mM Tris-HCl, 100mM NaCl, 10mM EDTA, pH-8.0). The harvested cells were homogenized by sonication for 10 minutes (5sec pulse, 5sec interval) at 4°C with a pulse of 40% amplitude using Vibra cell Ultrasonic Processor (Sonics, California, USA). The cell lysate was then centrifuged at 4°C and supernatant was collected for further experiments. The fluorescence intensity of the supernatant was measured by recording the fluorescence at 507nm (with excitation at 488nm for strains carrying *gfp_{mut1}* plasmid) using Spectrofluorometer by adjusting band width of 5 and path length of 1cm. The fluorescence intensity of pSG1151 was compared with that of respective strain (pDSA1) at different time intervals.

2.8 GFP expression analysis by fluorescence microscopy:

The bacterial cells were monitored at different time intervals for the fluorescence at 507nm (with excitation at 488nm for strain carrying *gfp_{mut1}* plasmid) by Fluorescent microscopy using Fluorescent microscope (Nikon, Japan).

2.9 Cloning and expression of peptidase under *cry* regulatory elements

The primers with restriction enzyme site added to the 5' end (**PDFP SalI** 5' - ACG CGT CGA CGT ATG AAA AAA GTA TCA ATT CGG - 3' and **PDRP XbaI** 5 - TGC TCTAGA TTA TTT AAT TTC GAA TTC TCC - 3') were designed to amplify the region spanning open reading frame for cold peptidase. PCR amplification of the peptidase gene was done in a Thermocycler (Agilent Technologies, USA) with the procedure described in the section 3.3.6 at 62°C annealing temperature. The PCR-amplified product was purified and digested with restriction enzymes; HindIII and XbaI (NEB, USA) at 37°C for 4 hours. pSG1151 and pSG1151 D1 plasmids were digested overnight with the aforementioned restriction enzymes at 37°C. The digested PCR product and vector were ligated using T4 DNA Ligase (NEB, USA) at 16°C for 16 hours and transformed into *DH5a* strain of *E. coli*. The transformed cells were plated on ampicillin (100 µg/ml) supplemented LB Agar plates. After incubation at 37°C for 18 hours, clones with successful recombination showing ampicillin resistance were selected and multiplied. The cell free supernatant of *Bacillus sp.* S1DI 10 and recombinant pDSA1CP were also collected at different time points to analyze the expression of peptidase by SDS-PAGE and by measuring the specific activity of the extracellular peptidase.

3. Results

3.1 Cloning of terminator region of *cry* gene:

The amplification of terminator region of *cry* gene from *B. thuringiensis subsp. kurstaki* HD-1 resulted in a ~209bp PCR product. Figure 1 depicts the PCR product of terminator region of *cry* gene. The plasmid map of pSG1151 with restriction enzymes selected for cloning of terminator and promoter region are shown in Figure 2. S1 depicts the digested products from the transformed pSG1151T plasmid.

3.2 Cloning of promoter region of *cry* gene:

The promoter region of the *cry* gene was amplified in such a way to give three different reading frames. The amplification of promoter region of *cry* gene from *B. thuringiensis subsp. kurstaki* HD-1 resulted in a ~350bp PCR product for all three frames. Figure 3 depicts the PCR product of promoter region of *cry* gene for all three frames. S2 depicts the digested products from the transformed plasmids. The constructs carrying three frames were named as pDSA1, pDSA2 and pDSA3 for *gfp_{mut1}*. Figure 4 represents the structural organizations of these constructs.

3.3 GFP expression analysis by fluorescence spectroscopy and microscopy:

The expression of GFP under the promoter/terminator expression system was evaluated by checking the expression of gfp_{mut1} under the same promoter/terminator. The results obtained from the fluorescence spectroscopic and microscopic studies of pSG1151 were compared with the modified constructs of gfp_{mut1} . The GFP content of pSG1151 and three constructs; pDSA1, pDSA2 and pDSA3 were compared, out of which pDSA1 had higher GFP content and gave maximum fluorescence (Figure 5 and 6). The fluorescent intensity of pSG1151 and pDSA1 was also compared up to 72 hours at a time interval of 6 hours. Both the sample showed detectable fluorescence which increased with time up to 12 hours, but there was a much higher increase in the fluorescence of pDSA1 as compared to pSG1151 that enhanced slightly with time (Figure 7 and 8).

3.4 Cloning and expression of peptidase:

In order to study the expression of an enzyme under *cry* promoter and terminator, cold peptidase gene previously isolated from *Bacillus sp.* S1DI 10 (Singh et al., 2019) was cloned in the constructed vectors. The amplification of cold peptidase gene from S1DI 10 resulted in a ~2400bp PCR product. Figure 9 depicts the PCR product of peptidase gene and Figure 10 represents the plasmid map of pDSA1 showing restriction enzymes selected for cloning of cold peptidase. S3 depicts the digested products from the transformed pSG1151CP and pDSA1CP plasmids. The transformed *E. coli DH5a* colonies were patched on skim milk agar plates and the clones of pSG1151CP and pDSA1CP were compared on the basis of zone of clearance. The zone of clearance of pDSA1CP was much higher as compared to that of pSG1151CP and wild *Bacillus sp.* S1DI 10 (Figure 11). The expression of peptidase in pDSA1CP was also compared to the expression of peptidase in wild *Bacillus sp.* S1DI 10. The cell free supernatant of *Bacillus sp.* S1DI 10 and recombinant pDSA1CP collected at different time points was assayed for the specific activity of the extracellular peptidase. There was 2.7 fold higher protease activity of in recombinant pDSA1CP than that of wild *Bacillus sp.* S1DI 10 at 72 hours (Figure 12). The expression of extracellular peptidase from pDSA1CP at different time intervals was also higher than that of wild *Bacillus sp.* S1DI 10 as observed from the SDS-Page (Figure 13).

4. Discussion

Bacterial genes contain a specific regulatory DNA sequence upstream of a gene called the promoter which acts as a binding site for RNA Polymerase for the initiation of transcription. (Browning and Busby, 2004, Haugen et al., 2008). Mendoza-Vargas et al., (2009) reported that the expressions of approximately 800 genes in *E. coli* are regulated by the upstream promoter regions. In the present study, a promoter region from *B. thurigiensis kurstaki* comprising ~350 bp and a terminator region of ~209 bp were cloned upstream and downstream, respectively, of gfp_{mut1} in the plasmid vector pSG1151. The expression of GFP under the promoter/terminator expression system was evaluated by checking the expression of gfp_{mut1} by fluorescence spectroscopy and microscopy. The results obtained suggested that the expression of gfp_{mut1} was upregulated under the influence of the promoter/terminator based expression system. The promoter region of various *cry* genes consists of two overlapping regions which are active during different time points from log phase to sporulation phase resulting in the overproduction of the

crystal protein (Sedlak et al., 1998). The expression of *cry1Ac* gene in a construct carrying *cry1Ac* gene under the promoter was much higher than the mutant strain carrying *cry1Ac* gene without the promoter (Perez-Garcia *et al.*, 2010). A previous study conducted by Du et al., (2012) reported that the expression of *cry8Ea1* gene is regulated by two promoters upstream to the gene. Wang et al., (2013) reported a promoter region which regulates the expression of proteins in *B. thuringiensis* and an upstream leader sequence which helps in stabilizing the downstream mRNA. In addition, the expression of *cry* genes is also regulated by certain other factors. Doruk et al., (2013) reported significantly higher production of endotoxin by the recombinant strain having polyphosphate kinase gene as compared to the wild strain.

In order to check the expression of a heterologous bacterial gene encoding for an enzyme in the promoter/terminator expression system, cold peptidase enzyme isolated from a *Bacillus sp* S1D1 10 was used in our expression system. (Singh et al., 2019). Enzyme purification and characterization are essential basic requirements for effective industrial application of enzymes. The strength of the promoter/terminator expression system was further analysed by checking the expression of an extracellular cold peptidase under the influence of the promoter and terminator. The cell free supernatant of *Bacillus sp.* S1DI 10 and recombinant pDSA1CP collected at different time points was assayed for the specific activity of the extracellular peptidase. The results revealed that there was 2.7 fold higher activity (at 72 hours) of pDSA1CP than that of wild *Bacillus sp.* S1DI 10. The expression and activity of an extracellular lipase increased significantly under the regulation of P₄₃ and P_{AE} promoters in *B. subtilis* (Ma et al., 2018). Lee et al., (2010) reported the expression of both *lacZ* and an extracellular enzyme cellulase was highly upregulated under the control of *cry3Aa* promoter region. Further, a slight modification in the same promoter region gave significantly higher expression than the expression under the wild type promoter. Ma et al., (2006) reported significantly higher activity of an intracellular lipase when expressed under the control of a strong promoter in *B. subtilis*. The expression of an extracellular enzyme pullulanase was enhanced in the recombinant strain of *B. subtilis* under the regulation of a strong promoter (Song et al., 2016). The expression and activity of an extracellular lipase increased significantly under the regulation of P₄₃ and P_{AE} promoters in *B. subtilis* (Ma et al., 2018).

5. Conclusion

The promoter/terminator based expression system constructed in the present work can be used to express the industrially important proteins which will further cut the cost of inducer, as the novel approach developed for this expression system does not require induction by an inducer.

Declarations

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Author Contributions:

Conceived and designed the experiments: AKK, SMT. Performed the experiments: DS, Samiksha. Analysed the data: DS, Samiksha, SMT and AKK. Wrote the manuscript: DS and Samiksha.

Compliance with Ethical Standards

Conflict of interest

The authors declare that there is no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

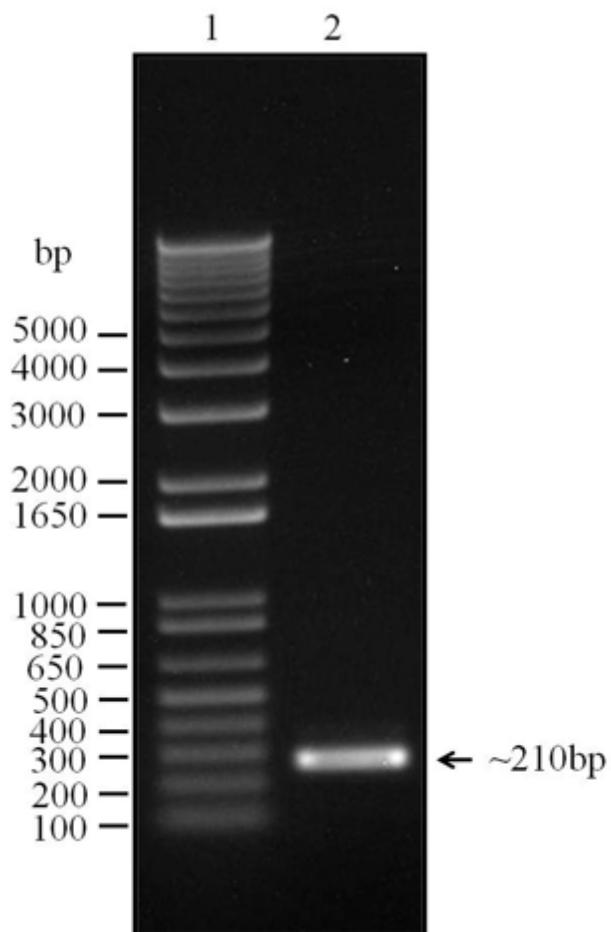


Figure 1

PCR amplified product of terminator region of *B. thuringiensis* subsp. *kurstaki* (Lane 1 – 1 kb DNA Ladder, Lane 2 – Terminator region)

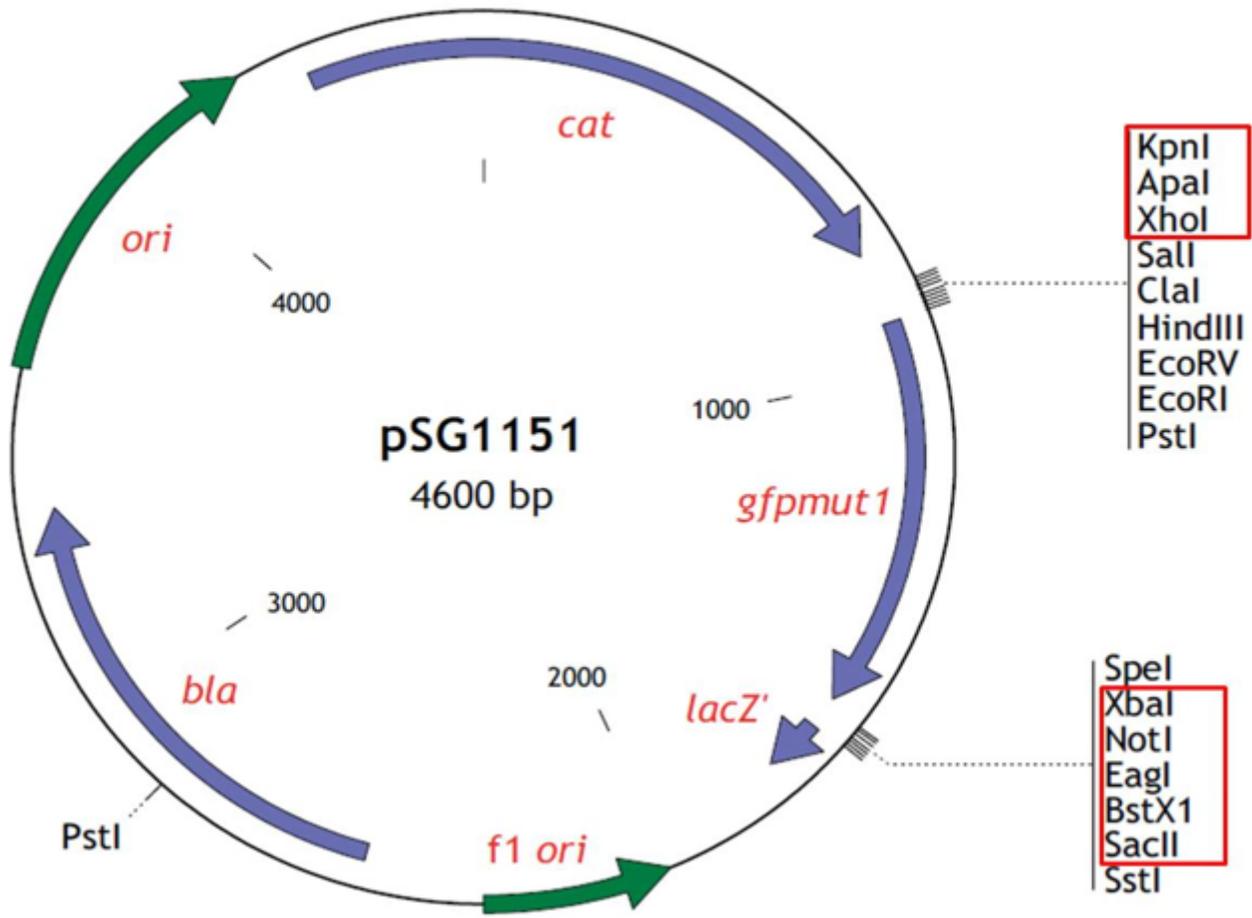


Figure 2

Plasmid map of pSG1151 (promoter region was cloned between KpnI and XhoI, terminator region was cloned between XbaI and SacII)

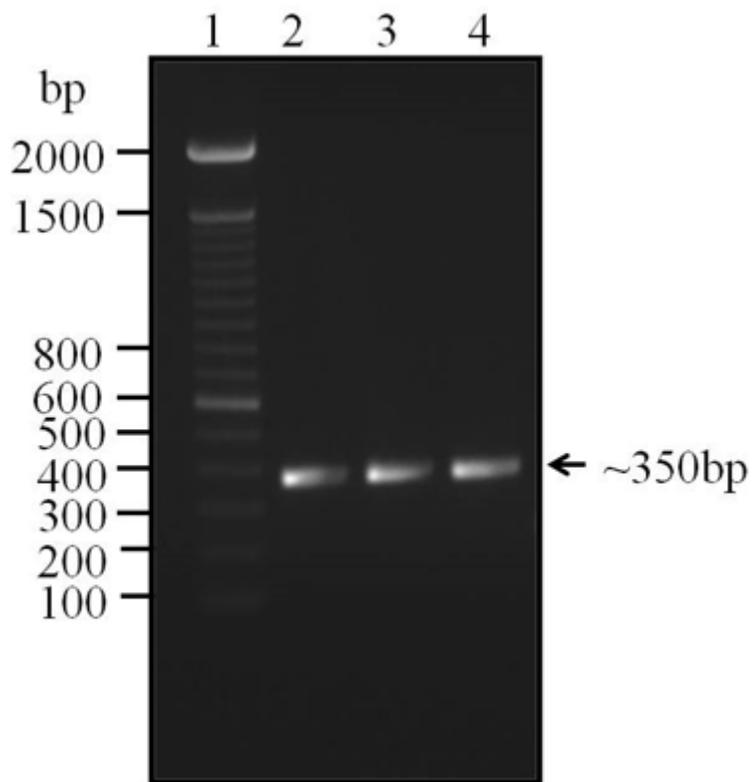


Figure 3

PCR amplification of promoter sequence for pSG1151 (Lane 1 – 100 bp DNA Ladder, Lane 2 – Promoter 1, Lane 3 – Promoter 2, Lane 4 – Promoter 3)

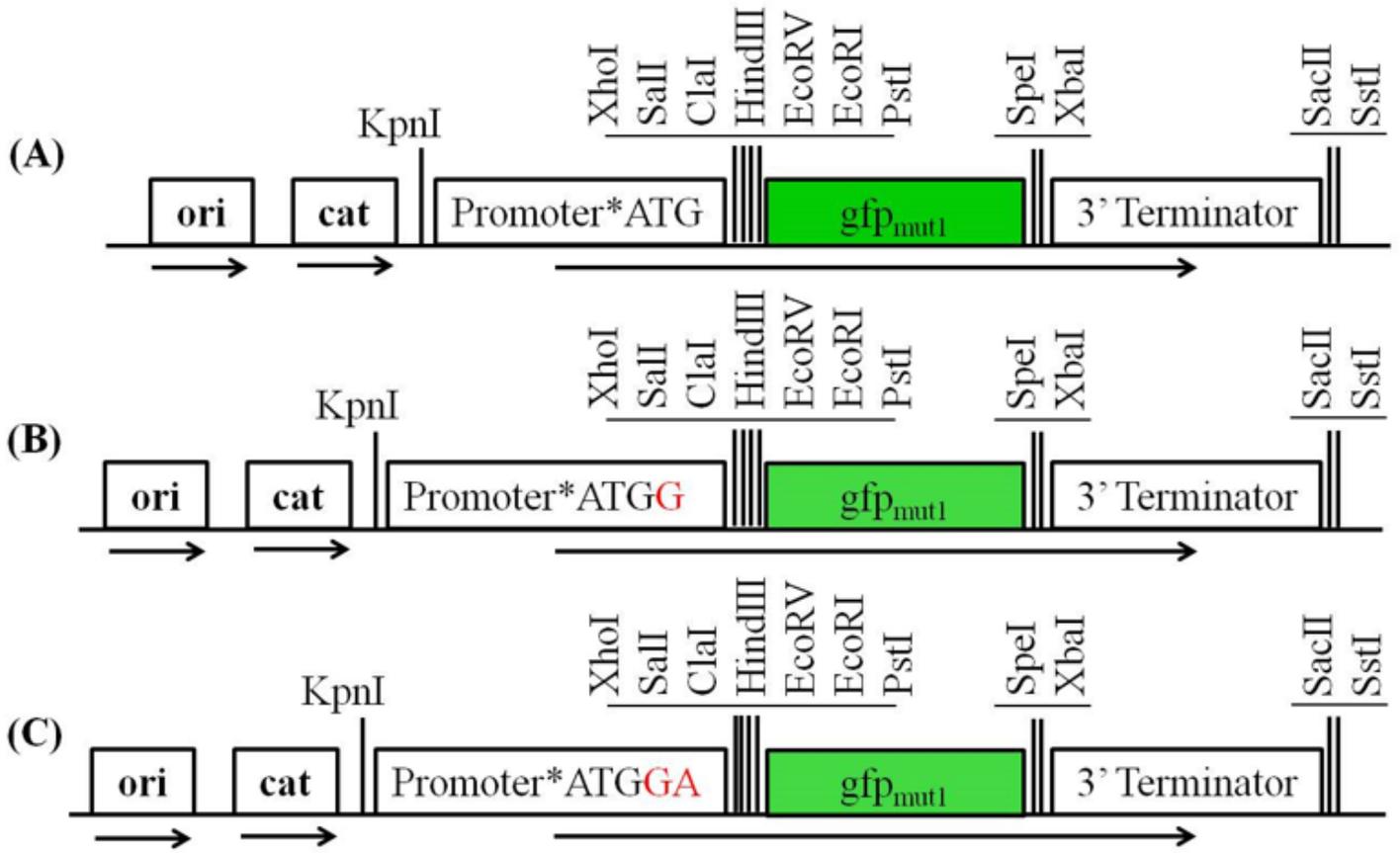


Figure 4

Structural organization of (A) pDSA1, (B) pDSA2 and (C) pDSA3.

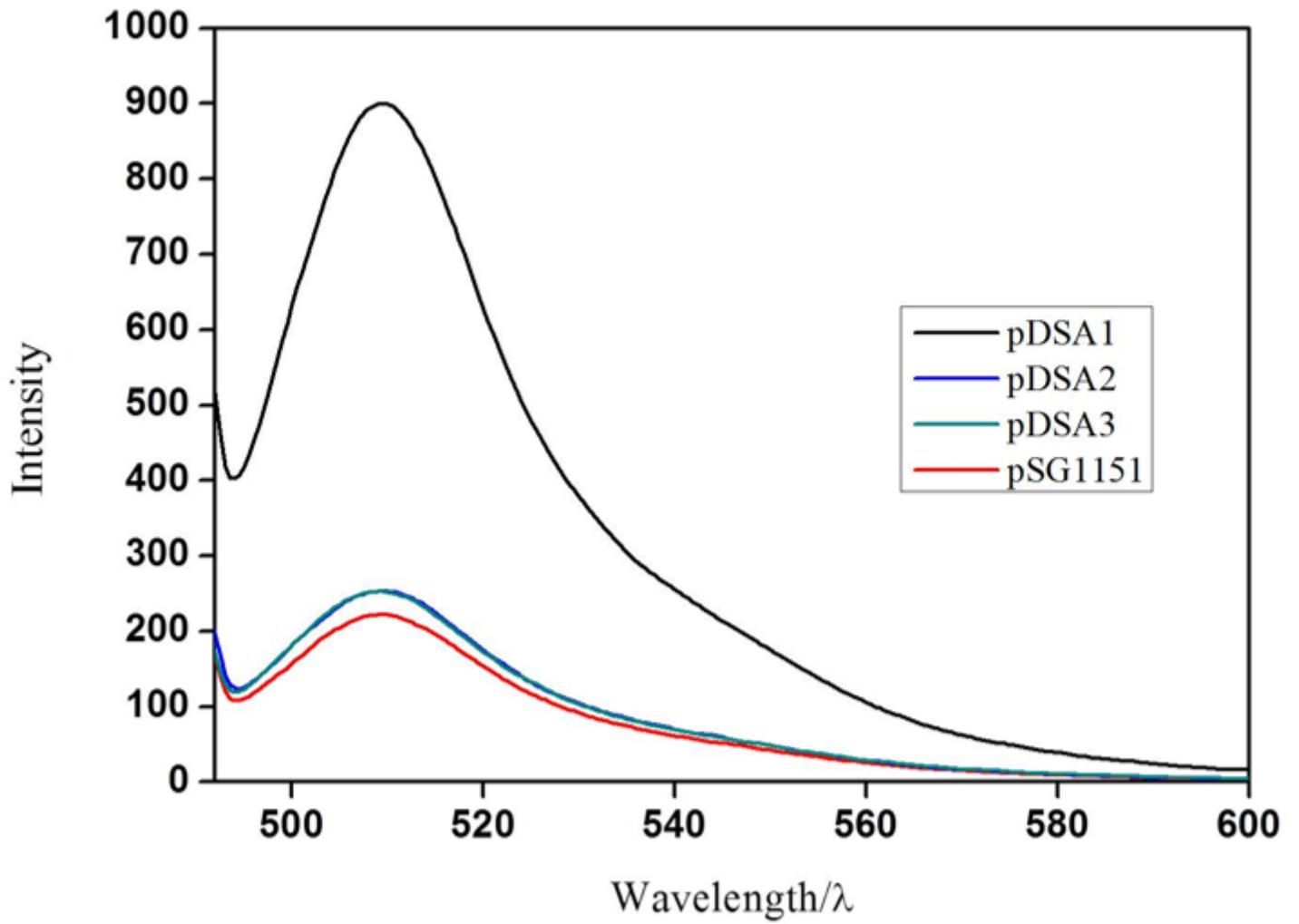


Figure 5

Fluorescence spectroscopy graphs showing fluorescence intensity of different constructs carrying gfpmut1

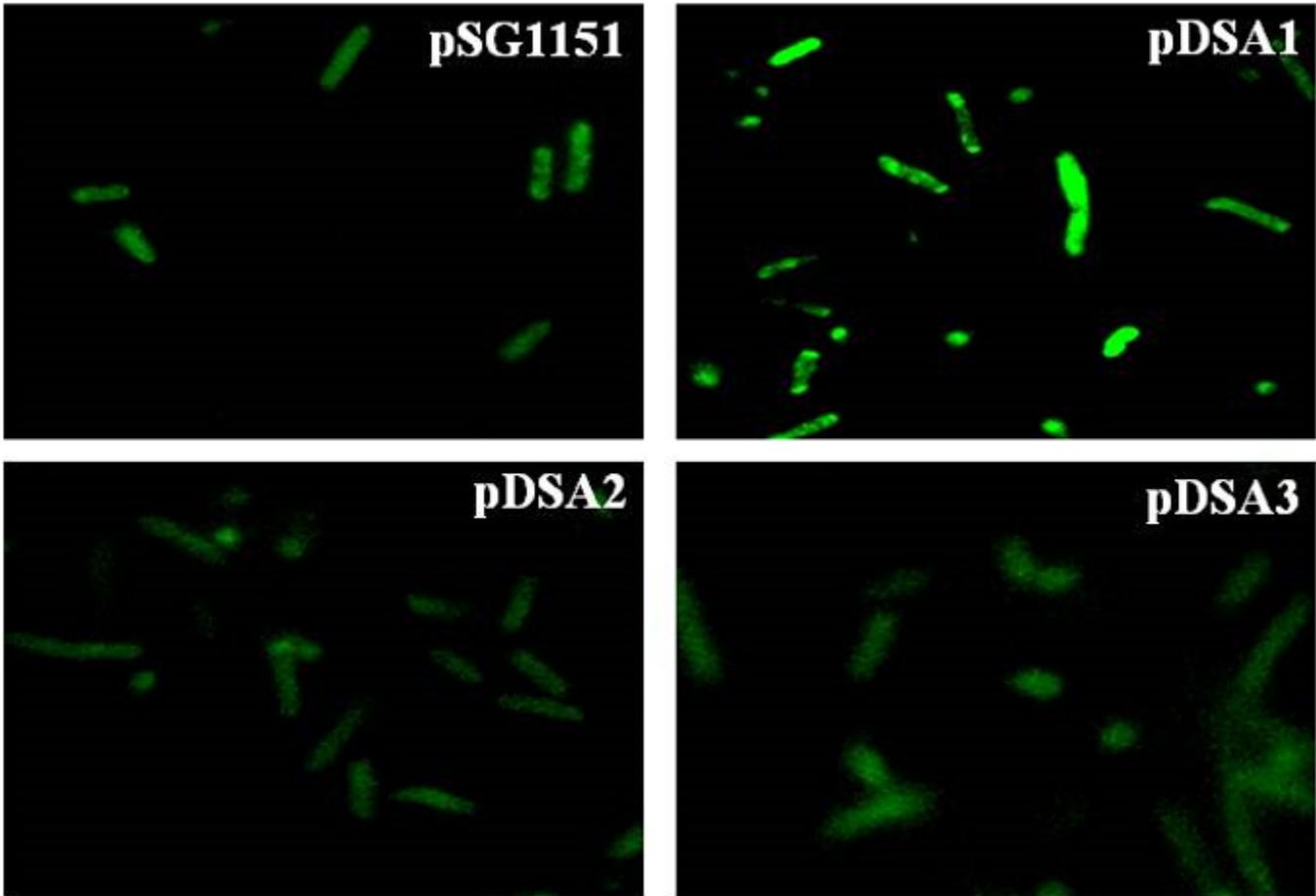


Figure 6

Fluorescence images (100X) of *gfpmut1* expressed by different constructs

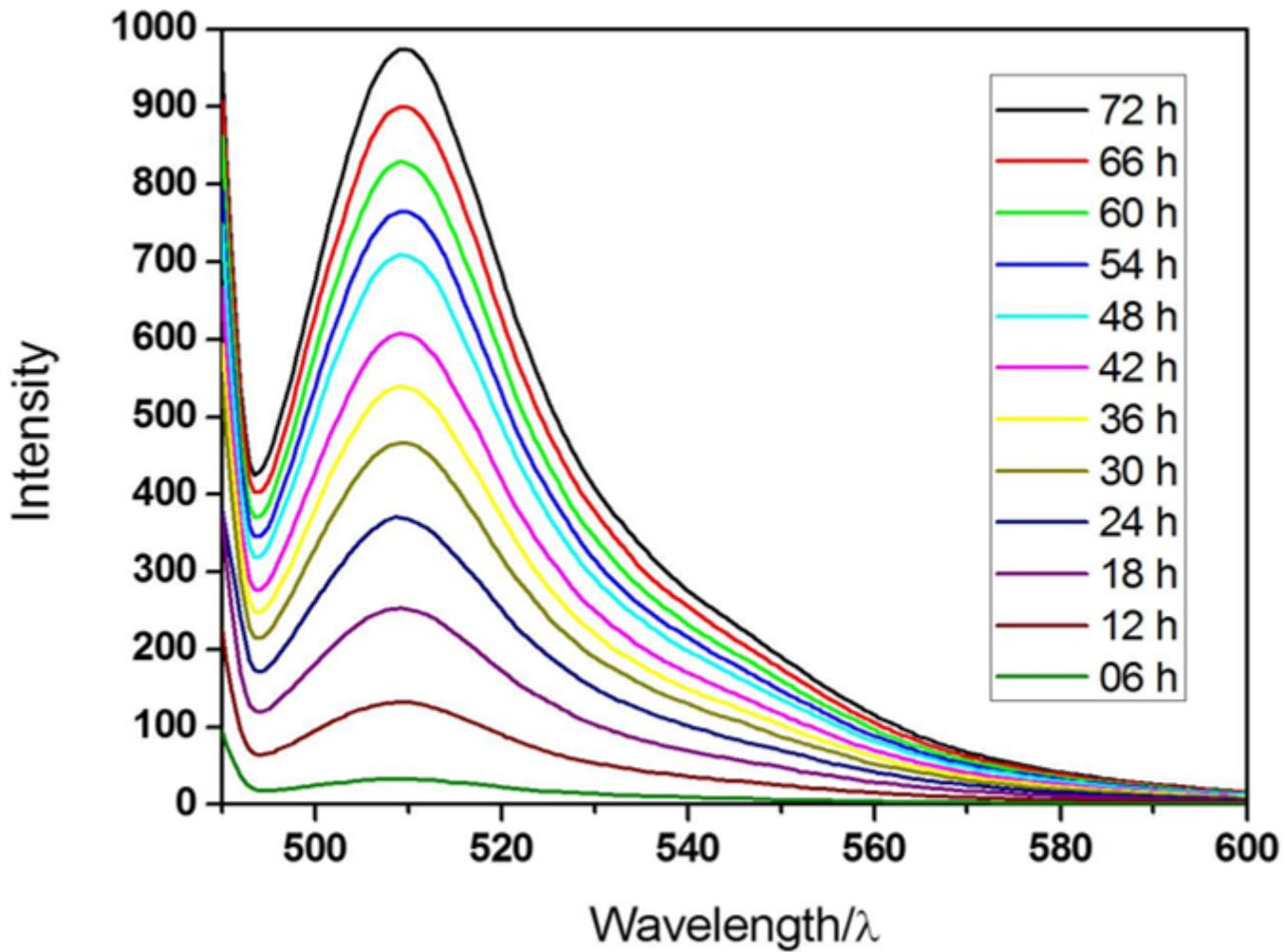


Figure 7

Fluorescence spectroscopy graphs showing fluorescence intensity of pDSA1 at different time intervals

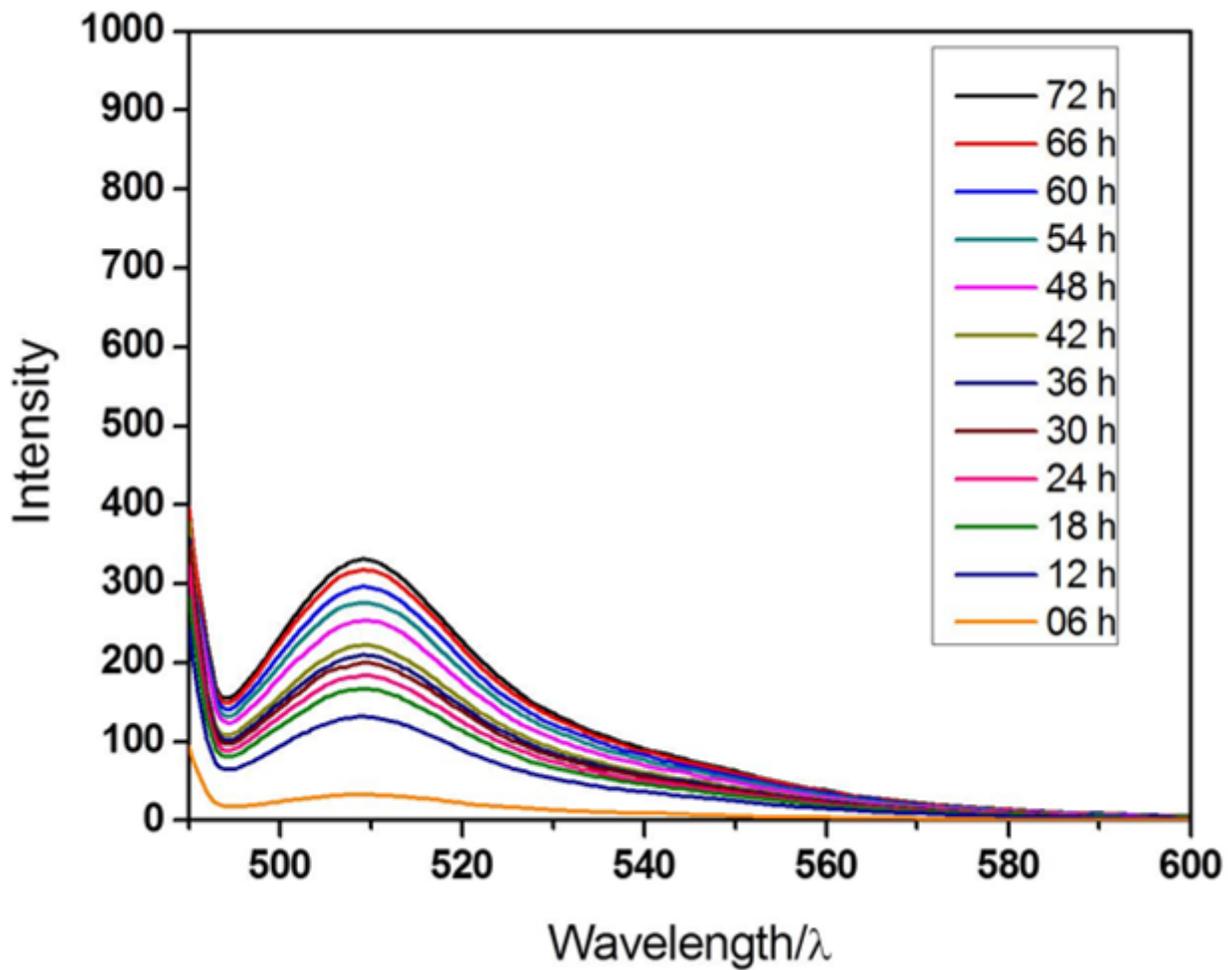


Figure 8

Fluorescence spectroscopy graphs showing fluorescence intensity of pSG1151 at different time intervals

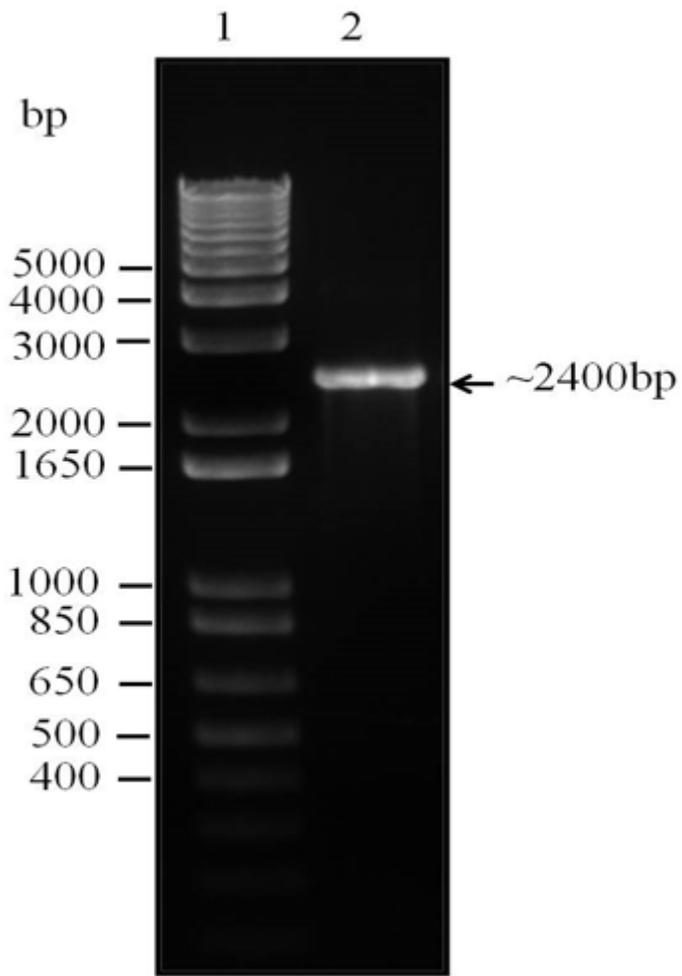


Figure 9

PCR amplified cold peptidase gene. (Lane 1 - 1 kb DNA Ladder, Lane 2 - peptidase gene)

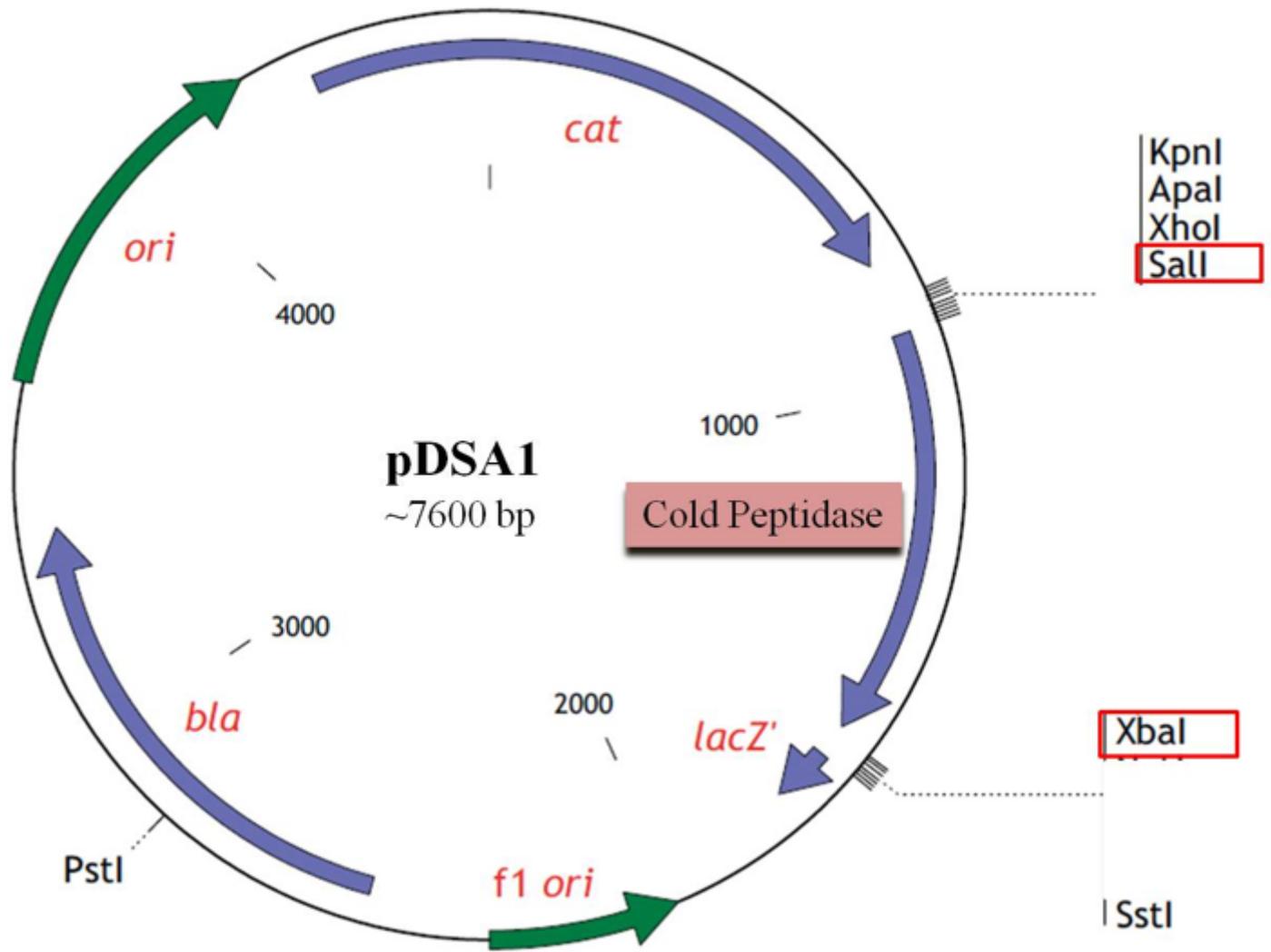


Figure 10

Plasmid map of pDSA1 (cold peptidase was cloned between Sall and XbaI)



Figure 11

Comparison of zone of clearance. (1 – *Bacillus* sp. S1DI 10, 2 – pDSA1CP, 3 – *E. coli*, 4 – pSG1151CP)

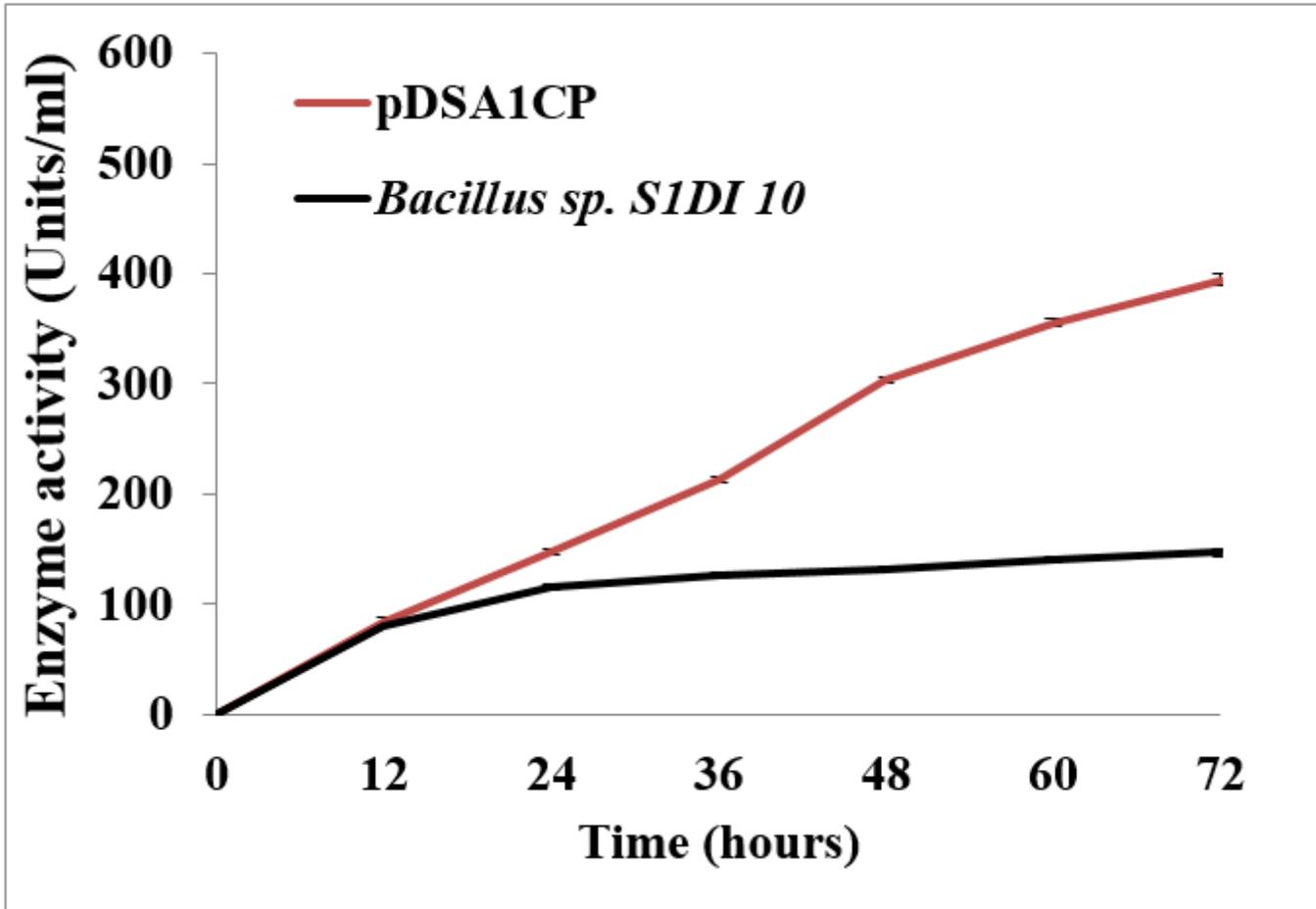


Figure 12

Peptidase production by pDSA1CP and *Bacillus sp. S1DI 10* at different time intervals.

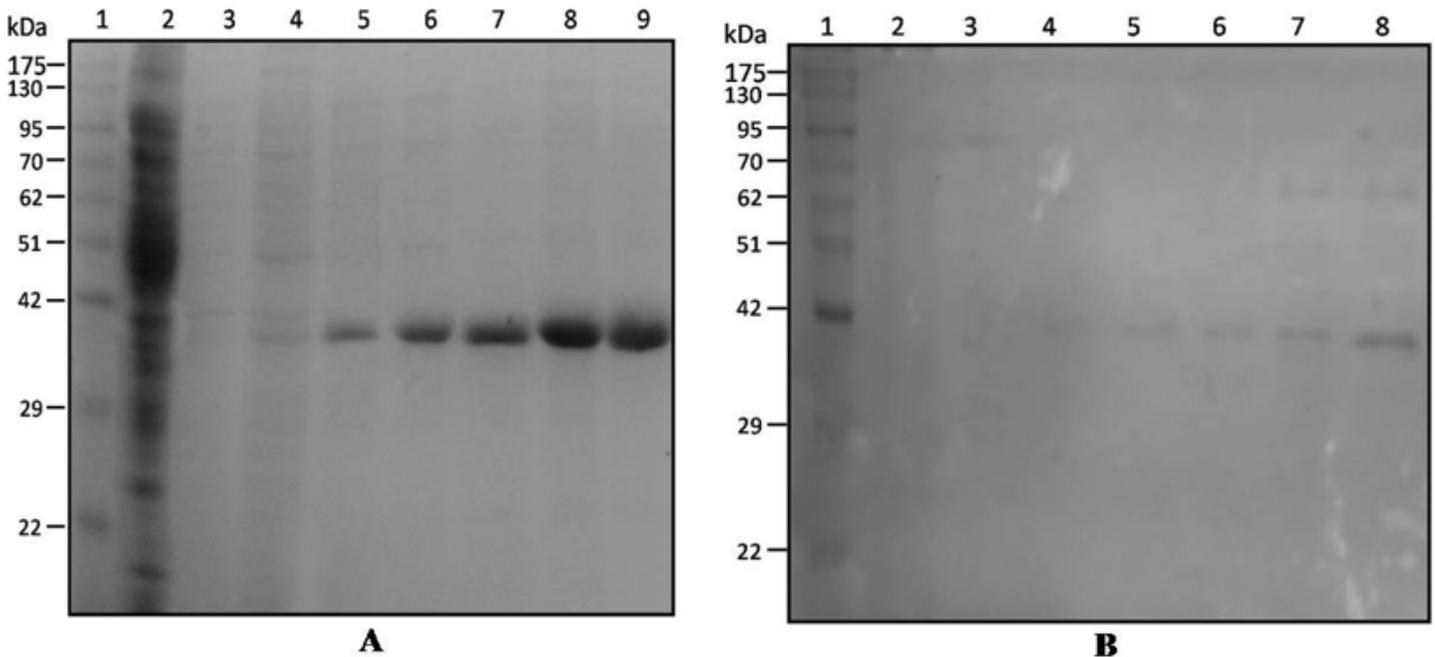


Figure 13

(A) SDS PAGE analysis of cold peptidase expressed in pSA1CP at different time intervals. (Lane 1 - Protein Ladder, Lane 2 - Negative Control (E. coli BL21DE3), Lane 3 - Total protein at 6h, Lane 4 - Total protein at 12h, Lane 5 - Total protein at 24h, Lane 6 - Total protein at 36h, Lane 7 - Total protein at 48h, Lane 8 - Total protein at 60h, Lane 9 - Total protein at 72h). (B) SDS PAGE analysis of cold peptidase expressed in Bacillus sp. S1DI 10 at different time intervals. (Lane 1 - Protein Ladder, Lane 2 - Total protein at 6h, Lane 3 - Total protein at 12h, Lane 4 - Total protein at 24h, Lane 5 - Total protein at 36h, Lane 6 - Total protein at 48h, Lane 7 - Total protein at 60h, Lane 8 - Total protein at 72h).

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