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# Design, construction and evaluation of protein expression systems in Synechocystis PCC 6803.

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**Design, Construction and evaluation of protein expression system in different bacteria**

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## Abstract

Plasmids are widely used as expression vectors for the production of industrially important recombinant protein and are also the basis for molecular biological research. The feature of the plasmid and features of the expression control system have a great influence on protein production. The plasmid copy number and the promoter and 5'UTR (untranslated region) plays a key role in controlling the amount of transcript produced from a gene. If there is too much transcript produced, this can cause a metabolic burden to the protein-expressing cell. Therefore, it is necessary to the promoter and 5'UTR, as well as growth phase of the cells that optimizes protein expression. This thesis focuses on the 5'UTR that is expressed under the control of inducible promoter system Xyls/Pm, which has previously shown to play an important role in controlling gene expression[1]. An alternative to utilizing *E.coli* as a protein expression system, are other prokaryotic organism that offer advantages. This thesis explores the protein expression in different bacteria (*Pseudomonas putida* KT2440, pSM6, pSM32 and *Synechocystis* PCC 6803) at varying temperature using the fluorescent protein m-cherry as a reporter gene and 0.1mM m-toulate as inducer of the Xyls/*Pm* promoter.

## Nomenclature

bp	basepair
CHO	
DNA	deoxyribonucleic acid
<i>E.coli</i>	
FDA	Food and Drug Administration
Km <sup>r</sup>	kanamycin resistance
OriT	Origin of transfer
OriV	Origin of vegetative DNA replication
nt	nucleotide
PCR	polymerase chain reaction
RNA	ribonucleic acid
rDNA	recombinant DNA
SD	Shine-Dalgarno sequence
UTR	untranslated region
$\sigma$	sigma
$\alpha$	alpha
$\beta$	beta

# 1 Introduction

The term biotechnology was coined by Karl Ereky, a Hungarian Engineer, in 1919[2]. He is also known as “father” of biotechnology[60]. It has a longer history that people are practicing this technology for the benefit of humankind. The use of microbes for fermentation has been in practice for a long time and many techniques have developed from these ancient technologies into modern biotechnology like beer, wine and cheese production [59]. Biotechnology is “any technological application that uses biological systems, living organisms or derivatives thereof, to make or modify products or processes for specific use” [61].

In 1944 Oswald Avery, Colin McLeod and Maclyn McCarty recognize DNA as genetic material and by Hershey and Chase in 1952. This discovery has shaped the route of molecular biology. In 1953 James Watson and Francis Crick discover the molecular structure of DNA based on Rosalind Franklin X-ray structure analysis[3]. Different breakthrough creates important milestones in those emerging time in the history of modern biology. Similarly, the bacteria’s restriction endonucleases which can cleave foreign DNA at nonspecific sequences discovered by Werner Arber in early 1960’s [62] and sequence-specific restriction endonucleases in 1970 by Hamilton Smith uncovered the tools which finally lead to recombinant DNA technology [63].

In 1976 the first genetic engineered company, Genentech was founded where human insulin was produced by applying the genetic engineered techniques where the genes carrying the genetic code for human insulin, along with necessary regulatory mechanism was inserted into *E.coli* strain[4]. Frederick Sanger pioneered the technique to determine the sequence of Insulin[5]. Insulin became the first genetically engineered drug approved by the U.S. Food and Drug Administration (FDA). Similarly, Swiss researchers were able to produce pure interferon by introduced the human interferon gene into *E.coli* [6].

In 2010 researchers at the J. Craig Venter Institute were able to create self replicating synthetic bacteria cell[7] with a synthetic genome, which marks the maturing of molecular biology into synthetic biology.

## 1.1 Microbial expression hosts

Heterologous protein expression in model organism plays an important role in modern biology including medicine, industries and biotechnology. Expression platforms used for heterologous protein production are cells or organisms. The known expression system for use in different hosts available today range from bacteria, yeasts[8] and filamentous fungi [9], human or plants cells. There is no optimal expression system that can serve as host for the production of all types of recombinant protein production[10]. All of these expression systems have specific characteristics as well as limitation and drawbacks. In practical both economic and qualitative aspects have to be noticed while choosing these different platforms. Importantly, industrial production counts the use of inexpensive components and expect high economic yield from the product. On the other part quality of produced product is equally important, mainly in the pharmaceuticals production which is regulated under strict safety condition[11].

Due to voluminous physiological knowledge, well known methods, and advanced genetic tools prokaryotic expression platforms are widely used for the production of heterologous protein. The well known bacterium *E.coli* was first organism used to produce recombinant protein production and is of special interest since many decades. It has several characteristic as it has high growth rate, inexpensive growth media and can be cultivated at high cell densities. Also, large numbers of expression system are available for use in *E.coli* [12]. *E.coli* has many drawbacks and limitations as it cannot perform post translation modifications[10]. It is good to explore other hosts to avoid problems associated with *E.coli*, which can be gram positive, or gram negative bacteria or eukaryotic systems as fungi, yeasts, plants or human cells. *Pseudomonas fluorescens* which is gram negative bacterium is used for antibiotic production because of improved secretion capabilities[13]. Similarly, gram positive bacterium *Staphylococcus carnosus* is of interest which prevent proteolytic degradation which is problem for widely used *Bacillus subtilis* [11].

Although *E.coli* and *sacchoromyces cerevisiae* are widely used host organism in heterologous protein expression, but the importance of various host organism become clear because each organism offers specific advantages.



## **1.2 Bacterial hosts used in this study**

### **1.2.1 *Pseudomonas putida* KT2440**

*Pseudomonads* belong to gamma subclass of the Proteobacteria commonly found on soil and water habitats where there is presence of oxygen. It is gram-negative rod shaped, saprophytic bacterium which grows optimally between temperature 25°C-30°C. *Pseudomonas putida* KT 2440 has been certified as safety cloning host bacterium by Recombinant DNA Advisory committee[14]. Its genome has been sequenced in 2002 and containing genome size 6.18Mb which has an G+C content of 61.6% [14].

It has been known that this bacterium harbors a plasmid, which encodes enzymes that are necessary for toluene degradation, known as TOL plasmid or pWWO [15]. This bacterium is now of great interest in biodegradation processes [16] biocatalysts[17] and plant protection [18]. In previous studies, *Pseudomonas putida* KT2440 has been compared to *E.coli* K-12 for expression capabilities and it was reported that *P. putida* KT2440 yield upto 3.5 mg/l of pure, soluble, active antibody scFv fragment which was 2.5-4 times higher than those from equivalent cultures of *E.coli* K-12 expression host[19] which proves to be a good alternative and potential host for recombinant protein production.

### **1.2.2 Cold adapted *pseudomonas* bacteria (pSM26, pSM32)**

Prokaryotes (*E.coli*) and eukaryotes (yeasts and CHO cells) hosts are being utilized for the successful production of many recombinant proteins, however these conventional system have often been proved to be inefficient indeed[20]. It is not possible to achieve thermally labile protein in large scale at normal *E.coli* growth temperature as it has been noticed that host proteases results in degradation of product and nascent polypeptides misfolding results in protein aggregation and accumulation as insoluble inclusion bodies[21].

To address these problems utilizing *E.coli* as host for the production of recombinant protein lowering of cultivating temperature can be good alternative [22]. However, cultivating *E.coli* at

lower temperature effects in biomass production, which reduces the global process production.[20].Therefore, psychrophilic bacteria can be good alternative expression hosts for recombinant protein production at lower temperature. Cold adaptive bacteria are now of great interest for the study of protein evolution and molecular adaptation in extreme conditions[23]. In addition cold adapted enzymes from psychrophiles can be of great value in biotechnology industry[24].Here in this studies two different Psychrophilic *Pseudomonas* strains named pSM26 and pSM32 are used as an expression hosts. Both of these strains are gram negative bacteria found in deep sea and polar sea. (Rahmi on communication).

### 1.2.3 *Synechocystis* PCC 6803 as expression host

Cyanobacteria are oldest living organism on earth, believed to exists 3.5 billion years ago which are found in various part of the world[25]. Cyanobacteria are the only unique group of prokaryotes that can perform oxygenic photosynthesis like plants and of utilizing light energy to generate organic material and give off O<sub>2</sub> as by-product.([26].The unicellular cyanobacterium *Synechocystis* sp.PCC 6803 (hereafter *Synechocystis* 6803) is gram negative prokaryote [27] performing oxygenic photosynthesis isolated from an intertidal environment. *Synechocystis* was the first photoautotrophic organism to be sequence completely in 1996 [28] which make it as model organism for the study of photosynthesis, stress response, metabolism and gene regulation. *Synechosystis* 6803 are naturally transferable [29]. Transformation is achieved by uptake of DNA using type IV pili [30] followed by incorporation in host genome by homologous double recombination [31].There are several application to make this Cyanobacterium as model system for biological studies and fundamental research where regulable expression of gene in this host is useful[32].

*Synechocystis* 6803 is now of great interest organism in the field of biotechnological and industrial application. *Synecho* can tolerate high intensities light and have fast growing rate and

also it can grow non photo synthetically if glucose is provided as a carbon source. They are easily cultivable in desired quantities in media consisting of water, carbon dioxide, sunlight and some minerals. Therefore, they could be used as attractive alternative for the production of high value products. It could be applied by introducing the new biochemical pathways from other organism into *Synechosystis* 6803 or by altering metabolic flow through existing biochemical pathway[33].So, we are trying to explore whether it can be possible to establish *Xyls/Pm* system in this host.

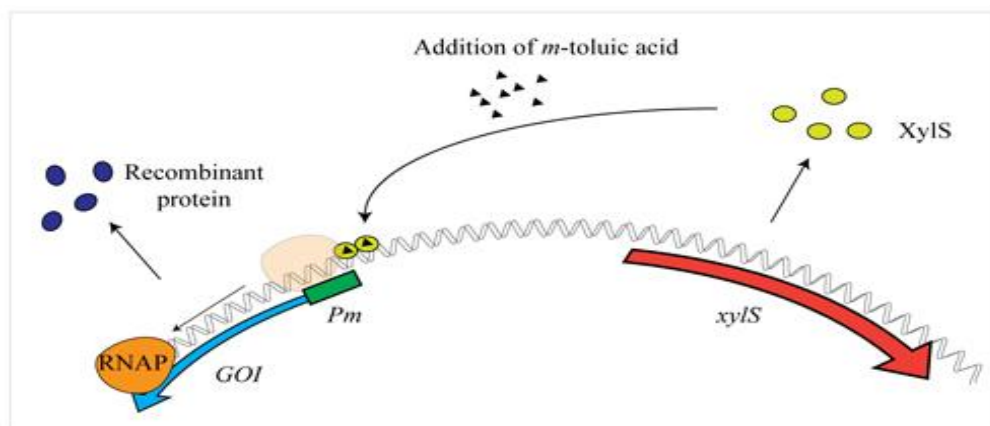
### **1.3 Genetic tools used in this study**

#### **1.3.1 3.1 RK2 Plasmid**

The replicon of RK2 plasmid which is broad host range plasmid was isolated from the bacterium *Klebsiella aerogenes* in 1969[34]. Rk2 plasmid, which belongs to the incompatibility group IncP $\alpha$ -1, replicates in many different gram negative bacteria and can be transfer by conjugation[35]. Minimal RK2 plasmid have been shown to be able to replicate in at least 58 Gram negative bacteria and in gram positive bacteria *Clavibacter xyli* [1].The copy number of RK2 plasmids has shown to be 4-7 per chromosome in *E.Coli* and 2-3 in *Pseudomonas putida*[36]. RK2 requires two essential components for replication. The *trans*-acting replication function *trfA* gene which encodes replication initiation protein and oriV, the *cis*-acting origin of vegetative DNA replication[37].

### 1.3.2 XylS/*Pm* system

The *Pm* promoter along with its cognate transcription activator XylS system is derived from the *Pseudomonas putida* pWWO plasmid[15]. Genes responsible for degradation of aromatic hydrocarbons are present in pWWO which is organized in two operons upper and lower(meta) operons [38-40]. The upper one converts xylenes and toluenes into alkylbenzoates or benzoates which are under control of *Pu* promoter[41] and meta or lower operon is responsible for degradation of carboxylic acids to krebs cycle intermediates which are under control of *Pm* promoter[42, 43]. The two regulatory proteins, XylS and XylR, control the expression of these two operons. The positive regulator XylR which is activated by substrates for the upper pathway, such as xylene controls the transcription from *Pu* [44]. Similarly, positive regulator XylS which is activated by substrates for the lower pathway such as *m*-toulate controls transcription from *Pm*. [45, 46]. The transcriptional factor XylS mediates inducibility of transcription from *Pm*.



**Figure 1: Positive control of XylS/*Pm* expression system. (Adapted from vectron biosolutions, 2010)**

Naturally, expression of XylS is controlled by two types of promoters i.e Ps1 and Ps2 ([1]).Ps2 is a  $\sigma_{70}$  dependent promoter where low constitutive levels of XylS gene is expressed in their inactive forms [47]. But if substitute benzoates are available, inactive XylS interacts with the inducer molecules and is activated, which increase expression from *Pm*. XylS is the transcription activator of AraC-XylS family, derived from TOL plasmid pWWO, *Pseudomonas putida*. It is composed of 321 amino acids with a C-terminal domain (CTD) involved in DNA binding and interaction with RNAP and an N-terminal domain required in effectors binding and protein dimerization[1].Also, depending on the growth phase *Pm* rely on two different  $\sigma$  factors, where



The 5'-UTR found together with the *Pm* promoter used in this study originates from the TOL plasmid. It is 32 nucleotides long between the transcriptional start and translational start site which contains putative SD-site 5'-GGAG-3'.



**Figure3: Figure 35'-UTR sequence. SD: Shine-Dalgarno sequence. Transcriptional and translational start sites are shown {derived from [1]}.**

Different strategies have been adopted to maximize translation initiation efficiency. In previous studies it has been reported that variants of this region led to maximize expression levels from *Pm* up to 20-fold stimulation[55]. It is found that the SD-site was unchanged and the variants showed the similar expression when combined with other promoters[55]. A test with different reporter genes explain a certain amount of gene-dependency for the stimulating impact of these variants on *Pm*[56].

## 2

### 3 Aim of study

*E.coli* is the best-studied and most extensively employed bacterium used for production of recombinant protein. However, previous work has revealed certain drawbacks and limitation inherent to *E.coli*. It has been known that every bacterial hosts offer specific advantages and varying protein expression characteristics. In particular, it is possible to induce protein expression in different hosts using the *Xyls/Pm* expression regulator system. Although *Xyls/Pm* and 5'UTR has been studied extensively in *E.coli* its functionality in different hosts is still unexplored. In this thesis protein expression, dependent on different variants of *Xyls/Pm* promoter and 5'UTR in the mesophilic *Pseudomonas putida* and psychrophilic *Pseudomonas* strains pSM26 and pSM32 were studied.

The main aim of the work was to determine *Xyls/Pm 5'UTR* functionality for the improvement of recombinant gene expression for *Pseudomonas putida*, and cold adapted *Pseudomonas strains* (*Psm26* and *Psm32*) bacteria. For this, a previously constructed library of *Xyls/Pm 5'UTR* library was evaluated in *Pseudomonas putida* and *Pseudomonas strains* pSM26 and pSM 32 at varying temperature(i.e. 30°C, 20°C and 10°C).In addition, it was also evaluated whether the *Xyls/pm* system could be established in *Synechocystis PCC 6803*.

4  
5

## 6 5. MATERIAL AND METHODS

### 6.1 5.1Media and Supplements

Luria-Bertani(LB) and Luria-Bertani Agar (LA)

10g/L	Tryptone
5g/L	Yeast Extract
5g/L	NaCl
20g/L	Agar bacteriological (For LA media)

Autoclave at 120°C for 20 minutes.

#### 6.1.1.1 *Synechocystis media (solid)*

BG 11-Solution	10ml
K <sub>2</sub> HPO <sub>4</sub> Solution	1ml

Na <sub>2</sub> CO <sub>3</sub> Solution	1ml
Ammonium Fe(III) Citrate Solution	1ml
1M TES-NaOH Ph 8.2	10ml
Sodium thiosulfate	3g
Agar	15 g

Added to 1 Litre distilled/filtered H<sub>2</sub>O.

#### **6.1.1.2 BG11 Liquid**

BG11 –Solution	10ml
K <sub>2</sub> HPO <sub>4</sub> Solution	1ml
Na <sub>2</sub> CO <sub>3</sub> Solution	1ml

Ammonium Fe (III) citrate solution 1ml

Added to 1 Litre distilled /filtered H<sub>2</sub>O.

#### **6.1.1.3**

##### **6.1.1.4 *Psi-medium***

5g/l	Yeast extract
20g/l	Trypton
5g/l	MgSO <sub>4</sub> .7H <sub>2</sub> O

Distilled water

pH adjusted to 7.6 with KOH.

Autoclave at 120°C for 20 minutes.

#### **6.1.1.5 TFB1**

2.94g/l	Potassium acetate (30mM)
12.1 g/l	Rubidium chloride (100mM)



1.945 g/l      Calcium chloride (10mM)  
15.70g/l      Manganese (II) chloride (50mM)  
150ml          Glycerol (15% v/v).  
Distilled water

The solution pH adjusted to 5.8 using diluted acetic acid. Sterilized by filtration. Stored at 4°C.

#### **6.1.1.6 TFB2**

2.1 g /l          MOPS (10mM)  
11.0g/l          Calcium chloride (75mM)  
1.21g/l              Rubidium chloride (10mm)  
150 ml          Glycerol (15% v/v).  
Distilled water

The solution pH adjusted to 6.5 using diluted NaOH. Sterilized by filtration. Stored at 4°C.

### **6.1.2 Antibiotics Stocks**

#### **6.1.2.1 Kanamycin**

50 mg/ml Kanamycin was dissolved in distilled water, filter sterilize using 0.2µl filters and aliquots 1ml in eppendrops and stored at -20°C.

#### **6.1.2.2 Chlormphenicol**

34 mg/ml Chlormphenicol was dissolved in 99% ethanol, filter sterilizes and stored at -20°C.

#### **6.1.2.3 m-Toulate Stock**

1M m-toulate was prepared using 1.3615 m-toulate in 10ml of 70% ethanol.

#### **6.1.2.4 Glycerol Stock solution**

60% Glycerol stock solution was prepared. Autoclave and store in room temperature.

## 6.2 5.2 Methods

### **Growth and cryopreservation of bacteria**

A sterile pipette tip was used to pick the colony on the plate or from a cell stock. This was transferred to desired medium in sterile tubes or sterile shake flasks with baffles. Antibiotics were added if required or for counter selection of desired strains. *Synechocystis* PCC 6803 was grown in Erlenmeyer flask in liquid BG-11 medium supplemented with 10 mM TES at 30°C and at 50 μmol photons m<sup>-2</sup> s<sup>-1</sup> white light. *Pseudomonas putida* KT2440 was grown in LB media at 30°C. Psychrophilic *pseudomonas* here pSM26 and pSM32 were grown in LB media at 20°C room temperature.

Cell cultures were grown for overnight and 40% sterile glycerol was mixed and incubated at -80°C for further uses.

### **6.3 Bioinformatics tool**

#### **Clone manager (Sci Ed Software)**

Clone manager (clone manager 6) is a program which is basically used in molecular studies for cloning simulation, primers designing, and alignment operation purposes. This program is used for drawing graphical maps, identifying ORFs and potential start codon.

### **6.4**

### **6.5 Bacterial Strains and Vectors**

The bacterial strain and the plasmids used in this study are described in the table below 1.

Plasmid map of the plasmids used in this study is shown in figure 4.

**Table 1 Plasmid map of the plasmids used in this study**

Species and Strains	properties	Source or Reference
<i>E.coli</i> DH5α	General cloning host	Bethesda Research Laboratories
<i>E.coli</i> S17_1	Conjugation strain	Simon et al. (1983)
<i>Ps.putida</i> KT2440	Expression host candidate	
<i>pSM26</i>	Host candidate	Tromso collection (unpublished)
<i>pSM32</i>	Host candidate	Tromso collection (unpublished)
<i>Synechosytis</i> PCC6803	Host candidate	University of Otago, Dunedin, New Zealand

## Plasmids

Plasmid	Properties	Source or Reference
pHH100	Pm>mCherry, Km <sup>r</sup> , XylS, trfA, oriV, oriT, trnBT1T2. 7819kb	NTNU (Rahmi)
Pm44	Identical to pHH100 except that a XbaI/PciI fragment (wild type <i>pm</i> sequence) was exchanged with <i>Pm</i> mutant sequence pm44 (figure). Km <sup>r</sup>	This study
Pm45	Identical to pHH100 except that a XbaI/PciI fragment (wild type <i>pm</i> sequence) was exchanged with <i>Pm</i> mutant sequence pm45 (figure). Km <sup>r</sup>	This study
Pm52	Identical to pHH100 except that a XbaI/PciI fragment (wild type <i>pm</i> sequence) was exchanged with <i>Pm</i> mutant sequence pm52 (figure). Km <sup>r</sup>	This study
Pm71	Identical to pHH100 except that a XbaI/PciI fragment (wild type <i>pm</i> sequence) was exchanged with <i>Pm</i> mutant sequence pm71 (figure). Km <sup>r</sup>	This study
Pm77	Identical to pHH100 except that a XbaI/PciI fragment (wild type <i>pm</i> sequence) was exchanged with <i>Pm</i> mutant sequence pm77 (figure). Km <sup>r</sup>	This study
Pm85	Identical to pHH100 except that a XbaI/PciI fragment (wild type <i>pm</i> sequence) was exchanged with <i>Pm</i> mutant sequence pm85 (figure). Km <sup>r</sup>	This study
Pm87	Identical to pHH100 except that a XbaI/PciI fragment (wild type <i>pm</i> sequence) was exchanged with <i>Pm</i> mutant sequence pm87 (figure). Km <sup>r</sup>	This study
UTR44	Identical to pHH100 except that a PciI/NdeI fragment(wild type 5'-UTR sequence) was exchanged with 5'-UTR mutant sequence UTR44 (figure). Km <sup>r</sup>	This study
UTR45	Identical to pHH100 except that a PciI/NdeI fragment (wild type 5'-UTR sequence) was exchanged with 5'-UTR mutant sequence UTR45 (figure). Km <sup>r</sup>	This study
UTR46	Identical to pHH100 except that a PciI/NdeI fragment (wild type 5'-UTR sequence) was exchanged with 5'-UTR mutant sequence UTR46 (figure). Km <sup>r</sup>	This study
UTR47	Identical to pHH100 except that a PciI/NdeI fragment (wild type 5'-UTR sequence) was exchanged with 5'-UTR mutant sequence UTR47(figure). Km <sup>r</sup>	This study
UTR69	Identical to pHH100 except that a PciI/NdeI fragment (wild type 5'-UTR sequence) was exchanged with 5'-UTR mutant sequence UTR69 (figure). Km <sup>r</sup>	This study
UTR73	Identical to pHH100 except that a PciI/NdeI fragment (wild type 5'-UTR sequence) was exchanged with 5'-UTR mutant sequence UTR73 (figure). Km <sup>r</sup>	This study
UTR77	Identical to pHH100 except that a PciI/NdeI fragment (wild type 5'-UTR sequence) was exchanged with 5'-UTR mutant sequence UTR77 (figure). Km <sup>r</sup>	This study
UTR84	Identical to pHH100 except that a PciI/NdeI fragment (wild type 5'-UTR sequence)	This study

was exchanged with 5'-UTR mutant sequence UTR84 (figure). Km<sup>r</sup>

UTR85	Identical to pHH100 except that a PciI/NdeI fragment (wild type 5'-UTR sequence) was exchanged with 5'-UTR mutant sequence UTR85 (figure). Km <sup>r</sup>	This study
UTR87	Identical to pHH100 except that a PciI/NdeI fragment (wild type 5'-UTR sequence) was exchanged with 5'-UTR mutant sequence UTR87 (figure). Km <sup>r</sup>	This study
UTR88	Identical to pHH100 except that a PciI/NdeI fragment (wild type 5'-UTR sequence) was exchanged with 5'-UTR mutant sequence UTR88 (figure). Km <sup>r</sup>	This study
PU44	Identical to pHH100 except that a XbaI/NdeI fragment (wild type <i>pm</i> 5'-UTR sequence) was exchanged with <i>pm</i> 5'-UTR mutant sequence pm44 (figure). Km <sup>r</sup>	This study
PU45	Identical to pHH100 except that a XbaI/NdeI fragment (wild type <i>pm</i> 5'-UTR sequence) was exchanged with <i>pm</i> 5'-UTR mutant sequence pm44 (figure). Km <sup>r</sup>	This study
PU77	Identical to pHH100 except that a XbaI/NdeI fragment (wild type <i>pm</i> 5'-UTR sequence) was exchanged with <i>pm</i> 5'-UTR mutant sequence pm44 (figure). Km <sup>r</sup>	This study
PU85	Identical to pHH100 except that a XbaI/NdeI fragment (wild type <i>pm</i> 5'-UTR sequence) was exchanged with <i>pm</i> 5'-UTR mutant sequence pm44 (figure). Km <sup>r</sup>	This study
PU87	Identical to pHH100 except that a XbaI/NdeI fragment (wild type <i>pm</i> 5'-UTR sequence) was exchanged with <i>pm</i> 5'-UTR mutant sequence pm44 (figure). Km <sup>r</sup>	This study

## 6.6 Oligonucleotides used for plasmid construction

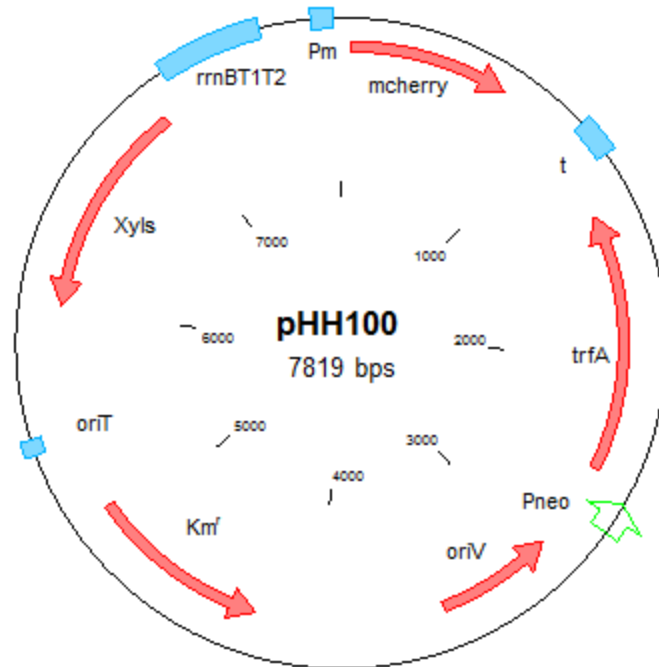
The synthetic oligonucleotide sequences were used to create plasmid constructs which was order from SIGMA-ALDRICH®. These sequences were identified in different *Pseudomonas* strains (*Pseudomonas putida*, *pSM26*, *pSM32*) obtained from screening. The forward and reverse strands were annealed and phosphorylates as described.

**Table 2A lists of the synthetic oligo nucleotides used in this study where sequence in 5'end to 3'end direction, length of oligonucleotide and restriction sites overhangs is shown.**

Sequences(5' to 3')	bp	Restriction sites
<b>Pm50-Fwd</b> CTAGAAAGACCTACCCCTCATGCTTAATGCAA	<b>32</b>	<b>XbaI, PciI</b>
<b>Pm50-Rev</b> CATGTTGCATAAAGCCTAAGGGGTAGGTCTTT	<b>32</b>	

Pm52-Fwd	CTAGAAAGGCTTACACCTTAGGCTTTATGCAA	32	XbaI, PciI
Pm52-Rev	CATGTTGCATAAAGCCTAAGGTGTAAGCCTTT	32	
Pm44-Fwd	CTAGAAAGGCCTACCCATTACGCTTTATGCAA	32	XbaI, PciI
Pm44-Rev	CATGTTGCATAAAGCGTAATGGGTAGGCCTTT	32	
Pm45-Fwd	CTAGAAAGGGCCTACCCCTTAGTCTTTATGGCAA	34	XbaI, PciI
Pm45-Rev	CATGTTGCATAAAGACTAAGGGGTAGGCCCTTT	34	
Pm71-Fwd	CTAGAAAGGCCACCTCTTAGGCTTTATGAA	31	XbaI, PciI
Pm71-Rev	CATGTTCATAAAGCCTAAGAGGTGGGCCTTT	31	
Pm77-Fwd	CTAGAAAAGCCTACCCCTTAGGCTTACTGCAA	32	XbaI, PciI
Pm77-Rev	CATGTTGCAGTAAGCCTAAGGGGTAGGCCTTT	32	
Pm87-Fwd	CTAGAAACTTCTACCCCTTATGCTTTATGCAA	32	XbaI, PciI
Pm87-Rev	CATGTTGCATAAAGCATAAGGGGTAGAAAGTTT	32	
Pm85-Fwd	CTAGAAAGTCCTACCCCTTAGGCTTTATGCAA	32	XbaI, PciI
Pm85-Rev	CATGTTGCATAAAGCCTAAGGGGTAGGACTTT	32	
UTR46-Fwd	CATGTACCCTAATAACGGATTCATGACCA	29	PciI, NdeI
UTR46-Rev	TATGGTCATGAATCCGTTATTAGGGTA	27	
UTR47-Fwd	CATGTTCAATAATTATGGAGTCATGAACA	29	PciI, NdeI
UTR47-Rev	TATGTTTCATGACTCCATAATTATTGAA	27	
UTR44-Fwd	CATGTCCAATATCAATGGGGTAATCAACA	29	PciI, NdeI
UTR44-Rev	TATGTTGATTACCCCATTTGATATTGGA	27	
UTR45-Fwd	CATGTACTACAACAAAGGAGGCATCAACA	29	PciI, NdeI
UTR45-Rev	TATGTTGATGCCTCCTTTGTTGTAGTA	27	
UTR69-Fwd	CATGTGCAATAATAATTGCGTCATGAACA	29	PciI, NdeI
UTR69-Rev	TATGTTTCATGACGCAATTATTATTGCA	27	
UTR73-Fwd	CATGTACAAAATCAATGGAGTAATGAACA	29	PciI, NdeI
UTR73-Rev	TATGTTCACTACTCCATTGATTTTGTA	27	
UTR77-Fwd	CATGTAAAATATTATGGATCATGAACA	27	PciI, NdeI
UTR77-Rev	TATGTTTCATGATCCATAATATTTTA	25	
UTR87-Fwd	CATGTACATTAATAATGGAGTCATGAACA	29	PciI, NdeI
UTR87-Rev	TATGTTTCATGACTCCATTATTAATGTA	27	
UTR84-Fwd	CATGTACTATTATAATGGATACATGAACA	29	PciI, NdeI
UTR84-Rev	TATGTTTCATGTATCCATTATAATAGTA	27	
UTR88-Fwd	CATGTACATTCATCAATAAGTCCTGAACA	29	PciI, NdeI
UTR88-Rev	TATGTTTCAGGACTTATTGATGAATGTA	27	
UTR85-Fwd	CATGTAAAACAGTAAAGGAGTCAAGAACA	29	PciI, NdeI
UTR85-Rev	TATGTTCTTGACTCCTTTACTGTTTAA	27	





**Figure 4 Map of plasmid pHH100**

Figure 4, showed that Map of plasmid pHH100 where mcherry as reporter gene, *pm*, positively regulated promoter; *xylS*, gene encoding *pm* activator; *km<sup>r</sup>*, kanamycin resistance; *trfA*, the gene coding essential replication protein; *oriV*, origin of vegetative replication; *oriT*, origin of transfer; *t*, bidirectional transcriptional terminator; *rrnBT1T2*, bidirectional transcriptional terminator. Details for the DNA sequences corresponding to the *pm* 5'-UTR are displayed above the map. SD; putative Shine-Dalgarno sequence. Transcriptional start site and translation start sites are indicated with an arrow. The following base substitution was identified in three different host from the screening of the library where HC44, HC45, HC46, HC47, HC50, HC52 was identified in *Pseudomonas putida*; HB69, HB71, HB73, HB77 in pSM26; HB84, HB85, HB87, HB88 in pSM32.

### 6.6.1

## 6.7 Phosphorylation and annealing of synthetic oligonucleotides

Synthetic oligonucleotides were ordered from SIGMA –ALDRICH®, which are synthesised as one forward strand and one reverse strands as single stranded DNA. It was important to anneal before use. Since, synthetic oligonucleotides do not have 5' phosphate so they need to be phosphorylated first with T4 polynucleotide kinase (PNK).

### Reaction mixture

- 7 $\mu$ L of forward oligonucleotide(100 $\mu$ mol/ $\mu$ L)
- 7 $\mu$ L of REVERSE oligonucleotide(100 $\mu$ mol/ $\mu$ L)
- 1.5 $\mu$ L T4 ligase buffer
- 0.8 $\mu$ L PNK

The reaction mixture was incubated at 37 $^{\circ}$ c for 30 minutes and 65 $^{\circ}$ c for 30 minutes for heat inactivation of enzyme. Then 4  $\mu$ L of 1 M NaCl was added to the reaction mixture and were allowed to anneal in Master cycler<sup>®</sup> PCR machine using programme named “Anneal\_Screening”.

### 6.7.1 Enzymes

All enzymes and buffers used in enzymatic reaction were ordered from New England Biolabs<sup>®</sup>inc

### 6.7.2 Sequencing

- 5 $\mu$ L of 80-100ng/ $\mu$ L of plasmid DNA (400-500ng total) was pipette in Eppendorf tube.
- If the concentration was dilute it was evaporate on heat block at 45 $^{\circ}$ c to increase concentration.
- 5 $\mu$ L of 5 $\mu$ M primer (552F) was added.
- Total 10  $\mu$ L of Plasmid DNA and primer was mixed in Eppendorf tube with myGATC label and was sending for sequencing.



### **6.7.3**

### **6.7.4**

### **6.7.5 Preparation of Competent cells**

1% of *E.coli* DH5 $\alpha$  competent cells grown overnight were inoculated in 10ml of Psi-medium. Then it was incubate at 37 $^{\circ}$ c 225 rpm until the OD600 reach exactly 0.4. Then cell culture was transferred on ice for 15 minutes, and was centrifuge at 4000rpm for 5 minutes at 4 $^{\circ}$ c. The supernatant was discarded and pellet was resuspended in 40ml cold TFB1.It was again incubate on ice for 5 minutes followed by centrifugation (4 $^{\circ}$ c, 4000rpm, 5 minutes).The suspension was carefully discarded and was resuspended in 3ml cold TFB2.The cell solution was aliquots in 100 $\mu$ l volumes sterile eppendrof tubes and sanp-frozen using liquid nitrogen.The aliquots were stored at -80 $^{\circ}$ c.

### **6.7.6 Heat-shock Transformation**

1 $\mu$ l of plasmid DNA was mixed with 100 $\mu$ l of DH5 $\alpha$  competent cells which was placed on ice. The mixtures were mixed gently and incubate back on ice for 30 minutes. Then cell were heat

shocked at 42°C for 45 second and incubate back on ice again for 3 minutes followed by adding 500µl of LB medium or SOC medium which was incubated at 37°C in shaking incubator for 1 hours. Then transformed cell were plated on LA plates with selective antibiotic for plasmid and incubate for overnight.

### **6.7.7 Conjugation**

*E. coli* S17\_1 cells were grown in the incubator at 37°C for 4 hours where *Pseudomonas putida* was grown in incubator at 30°C for 5 hours. Similarly, pSM26 and pSM32 was grown at 20°C room temperature using appropriate amount of LB to perform conjugation. 2 ml *E. coli* S17\_1 which contains target vector, and 2 ml psychrophilic *Pseudomonas* sp. (pSM26 and pSM32) or *Pseudomonas putida* were mixed by vortexing. The cells mixture was centrifuged at 4000 g for 5 min and supernatant was discarded. Pellet was allowed to dissolve in 100 µl LB and transferred to LB agar plates (without antibiotic) as one drop. Then plates were incubated 1 day at 30°C and 1 day at 20°C. After conjugation, each drop were streak out by sterile pipette tip from the agar plates and transferred into 1 ml of LB and diluted up to 10<sup>-2</sup>, 10<sup>-4</sup> and 10<sup>-6</sup> samples and was plated out on LA plates containing appropriate kanamycin and chloramphenicol. LA plates with kanamycin and chloramphenicol were incubated at 20°C for the selection of psychrophilic *Pseudomonas* sp. (*Pseudomonas putida*) from *E. coli* S17\_1.

### **Conjugation (*Synechocystis* species)**

At, first *E.coli* S.17.1 cell carrying plasmid DNA (pHH100) were grown to  $3.5 \times 10^8$  (O.D~1.0) and *Synechocystis* PCC6803 was grown to  $3.5 \times 10^8$  (OD~4).

#### Washing

*E.coli* cells were spin down at 9000g for 10 minutes and were resuspended in 10ml of BG11 solution and 5% of LB. Similarly, *Synechocystis* PCC6803 cells were centrifuging for 5000g for 10 minutes and was resuspended in 10 ml of BG11 medium and 5% of LB.

#### Combine

Then *E.coli* cells with 10 ml of BG11 medium and 5%LB was allowed to mix with 10ml of BG11 medium and 5%LB *Synechocystis* cells.

#### Incubate

The mixture was incubated at 30°C for 20 hours at 3000 lux (~60 $\mu$ , photons/m<sup>2</sup>\*s).

#### Plating out

0.1 ml of conjugate mixture was plated on BG11 plates. It was incubate at 30°C for 20 hours with continuous supply of light.

#### Adding of antibiotic

0.32 ml of 1g/L of kanamycin was injected beneath the BG11 agar plates using sterile micropipette. It was incubate at 30°C with supply of light in the incubator till growth.

### **6.7.8**

#### **6.7.9 Plasmid DNA purification**

Wizard ® plus sv minipreps DNA purification system.

- Single colonies were inoculated to sterile tubes containing 5 ml of LB using sterile tips and incubate overnight in shaker with required antibiotic.

- 5 ml of bacterial culture was taken in test tubes and centrifuge for 5 minutes at 10,000xg in tabletop centrifuge. The supernatant was discarded and excess media was removed by blotting the inverted tubes on a paper towel.
- Then 250µl of cell resuspension solution was added and resuspension of cell pellet was done by vortexing or pipetting. Then resuspended cells are transfer to a sterile 1.5ml microfuge tubes.
- 250µl of cell lysis solution was added and was mixed by inverting the tube 4 times. Cell suspension was incubated approximately 1-5 minutes until the cell suspension became clear.
- Then 10µl of alkaline protease solution was added and was mixed by inverting the tube 4 times. It was incubated for 5 minutes at room temperature.
- 350µl of neutralization solution was added and was mixed immediately by inverting the tube 4 times.
- The bacterial lysate was centrifuged at maximum speed around 14,000xg in a microcentrifuge for 10 minutes at room temperature.
- The cleared lysate was transferred to the prepared spin column by decanting without transferring any white precipitate.
- The supernatant was centrifuged at maximum speed in a microcentrifuge for 1 minute at room temperature. The spin column was removed from the tube and flow through was discarded from the collection tube. The spin column was reinserted into the collection tube.
- 750µl of column wash solution was added which was previously diluted with 95% ethanol, to the spin column.
- It was centrifuge at maximum speed in a microcentrifuge for 1 minute at room temperature. The spin column was remove from the tube and the flow through was discarded. The spin column was reinserted into the collection tube.
- The wash procedure was repeated using 250µl of column wash solution.
- It was centrifuge at maximum speed in a microcentrifuge for 2 minutes at room temperature.

- The spin column was transfer to a new, sterile 1.5ml microcentrifuge tube and plasmid was eluted by adding 100µl of nuclease free water to the spin column. It was allowed to centrifuge at maximum speed at room temperature for 1 minute in a microcentrifuge.
- After eluting the DNA, the assembly was removed from the 1.5ml microcentrifuge tube and the spin column was discarded and plasmid preparations was stored at stored at -20°C.

#### **6.7.10 QIAquick PCR purification kit protocol**

- 5 volumes of buffer PBI was added to 1 volume of the PCR sample and was mixed.
- QIAquick spin column was placed in a 2ml collection tube.
- The sample was pipette to QIAquick column to bind DNA and was centrifuged for 30-60 s.
- Flow through was discarded and QIA quick column was placed back into the same tube.
- 0.75 ml buffer PE was added to wash the QIAquick column and centrifuged for 30-60 s.
- Flow –through was discarded and the QIAquick column was placed back again in the same tube. The column was centrifuged for an additional 1 minutes.
- QIA quick column was placed back in a clean 1.5 ml microcentrifuge tube.
- DNA was eluted by adding 50 µl Buffer EB (10 mM Tris-CL, pH 8.5) to the centre of the QIA quick membrane and centrifuges the column for 1 minute.
- The elutated DNA was stored at-20°C.

#### **6.7.11 Restriction digestion**

Restriction digestion utilizes advantages of naturally occurring enzymes that can cleave DNA at specific sequence. There are various types of restriction enzymes, which helps to target wide variety of recognition sequence. Specific restriction enzymes have specific recognition sites in the nucleotide sequence. Mostly the sequences are four to six nucleotides long, but there may be

enzymes with longer recognition sites. The patterns of the cut differ between enzymes which either makes blunt or sticky ends. Restriction digestion helps to select the right gene fragments and vector.

Restriction mixtures made:

10  $\mu\text{l}$  isolated plasmid (concentration ranging from 20 – 150  $\mu\text{g}/\mu\text{l}$ )

1  $\mu\text{l}$  enzyme (NEB)

0.5  $\mu\text{l}$  BSA (if necessary) (NEB)

2  $\mu\text{l}$  10x concentrated buffer (1-4, specified for each enzyme) (NEB)

Sterile water to a total volume of 30  $\mu\text{l}$

If double digestion was carried and the buffers of the two enzymes are same, 1  $\mu\text{l}$  of each enzyme were added in the same mixture.

The restriction mixture was incubated 37°C for 2 hours. The restriction enzymes used in this study is shown in

### 6.7.12 Ligation

As restriction enzymes make specific cuts in DNA strands DNA-ligase helps to join the fragments. DNA ligation is enzyme which repairs nicks in double stranded DNA molecule.

Ligations mixture:

5  $\mu\text{l}$  Vector DNA

10  $\mu\text{l}$  Insert DNA (depending on the concentration)

0.5  $\mu\text{l}$  DNA-ligase T4 (NEB)

2  $\mu\text{l}$  T4 DNA Ligase Reaction Buffer (NEB)

3  $\mu\text{l}$  Doubledistilled water

The total ligation mixture was made 20µl tubes were incubate at 16°C for overnight.

### **Measurement of m-cherry expression level using Tecan spectrophotometer**

A different variant of colonies were transferred from a fresh agar plate to a 96-well plate containing 100 µL LB with selective antibiotics (kanamycin) in each well. 23 different variants (three different hosts) were inoculated in 96-wells using sterile micropipette tips. The next day, replica was done from 96-well plate which was cultured overnight into three different sets of 96 well plates with kanamycin, three with (0.1mM m-toulate) and three without inducer. The 96-well plate were sealed with parafilm and incubated on a table shaker board at 30 °C, 20°C and 10°C, 1100 rpm for about 24 hours. Then measurement was done using Tecan spectrophotometer.

### **6.7.13 General overview of experimental procedure**

Previously, identified *Pm* and 5'-UTR variants was mixed by Rahmi. These complex library were screened on three different hosts, pSM26 and pSM32 and *Pseudomonas putida* KT2440 growing on LA plates with kanamycin and chlormphenicol for selection procedure. Randomly selected clones based on phenotype where m-cherry was used as reporter gene, 10 single clones from each host were selected and grow on LB media, isolate plasmid DNA and was send for sequencing. Sequencing result was interpreted by multiple sequence alignment. 14 sequence result were selected for further studies, where 8 different *pm* variants and 11 different 5'UTR variants was identified from different hosts. All of the identified different variants of *Pm* and 5'-UTR oligo nucleotides sequence were order form Sigma-Aldrich®. These oligo nucleotides sequences were phosphorylated and annealed as described. Then plasmid DNA pHH100 was used for cloning purposes where different variants of *Pm* and 5'-UTR and combination of both pm 5'-UTR was constructed with appropriate restriction enzymes and ligation was performed. In order to confirm correct insert these sequence were further send for sequencing using primer 552 F sequence shown above. Out of 24 variations, 7 variants of *pm*, 11 variants of 5'UTR and 5 variants of combinations of both pm 5'-UTR was confirmed from sequencing result. These 23

different combinations of plasmid were then transferred into *E.coli* S17.1. Then through conjugation these 23 different variations of combination were allowed to express in three different hosts i.e. *Pseudomonas putida*, psm26 and psm32 using 96 well plates. Next days, replica culture of 96 well plate was done in two plate one with 0.1mM m-toulate as inducer and other plate without inducer. Three sets of plates were prepared and incubated in varying temperature i.e. 30°C, 20°C and 10°C with continuous shaking at 1100 rpm. The measurement was carried by measuring fluorescence of m-cherry at OD600 using Tecan spectrophotometer in three different time variation i.e. 5 hours, 24 hours and 48 hours of induction using two biological replicas.

## 7

## 8 6.Result and discussion

### *Selection of Pm and 5'UTR variants identified in mesophilic and psychrophilic Pseudomonas bacteria for further studies.*

A plasmid *Pm* mutant library was constructed by Bakke et al[57], and 5'-UTR library was constructed by Lale et al[55]. The *pm* and 5'-UTR mutant variants library were mixed (Rahmi). We have screen complex *pm* 5'-UTR library utilizing *Pseudomonas putida*, *Psm26* and *Psm32* as hosts and m-cherry for selecting phenotype. While screening *pm* 5'UTR mutant library altogether 8 different *pm* variants, 11 different UTR variants and 5 combination of both *pm* 5'UTR variants were selected for further studies based on the phenotype and sequenced result. All of *pm* variants were none identical carrying point mutation one to four and one sequence carry deletion mutation near *pm* transcriptional initiation region (figure 5). Similarly, eleven non-identical 5'UTR variants carries one to seven point mutation where seven sequence carry point/deletion mutation within SD sequence (Figure 6). It was previously reported that mutation within SD region reduce the affinity to the ribosome which shows reduction in expression





**Figure 5** Pm variants identified in *Pseudomonas putida* (pm44, pm45, pm50, pm52); psm26 (pm71, pm 77) and psm32 (pm85, pm87). Base subjected to mutagenesis are written in lower case. Identical base are indicated by dots and deletion with dash. Transcriptional start site and unique restriction site is shown.

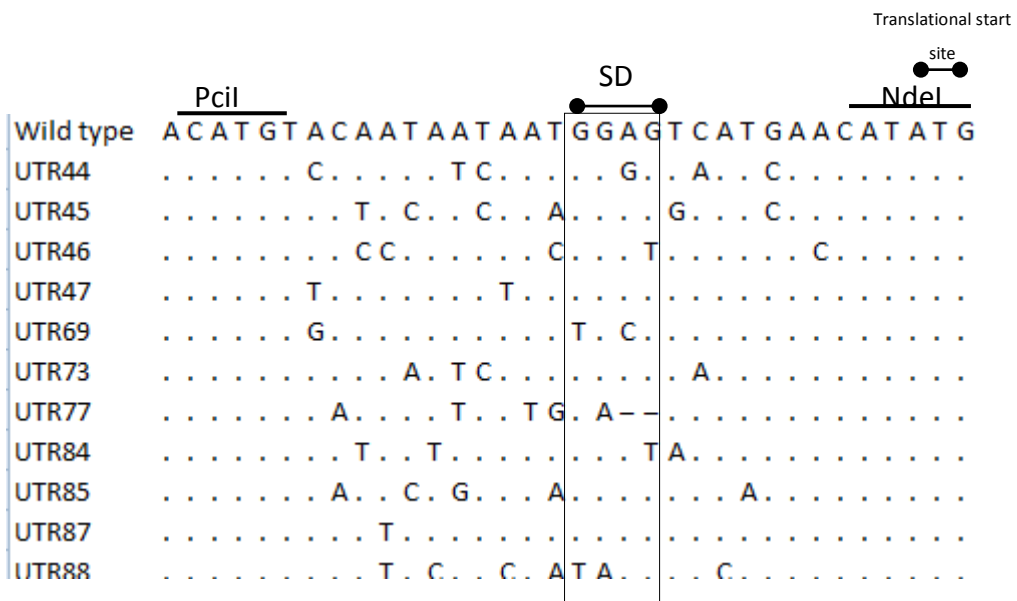


Figure6: UTR variants identified in *Pseudomonas putida* (UTR44, UTR45, UTR46, UTR47); *psm26* (UTR69, UTR73,UTR 77) and *psm32*(UTR84,UTR85, UTR87 ,UTR88). Base subjected to mutagenesis are written in lower case. Identical base are indicated by dots and deletion with dash. Putative Shine-Dalgarno sequence is marked as SD. Translational start site and unique restriction site is shown.

	Xbal	Transcriptional start site	SD	Translational start site	NdeI
Wild type	<u>TCTAGAA</u> AAGGCCTACCCCTTAGGCTTTATGCAACATGTACAATAATAATGGAGTCATGAACATATG				
PU 44	.....	..... A... C .....	..... C..... TC..... G.. A.. C.....		
PU 77	.....	..... A..... AC.....	..... A..... T.. TG.. A--.....		
PU 85	.....	..... T.....	..... A.. C.. G... A..... A.....		
PU 87	.....	..... CTT..... T.....	..... T.....		
PU45	.....	..... G..... T..... G.....	..... T.. C.. C.. A... G... C.....		

Figure7: *Pm* 5'UTR variants identified in *Pseudomonas putida* (PU44, PU45); *psm26* (PU77) and *psm32* (PU85, PU87). Base subjected to mutagenesis are written in lower case. Identical base are indicated by dots and deletion with dash. SD known as putative Shine-Dalgarno sequence is indicated. Transcriptional start site, translational start site and unique restriction site is shown.

**8.1.1.1 6.2 Performance of *Pm* variants displaying enhanced or reduced expression levels in *Pseudomonas putida* at varying temperature 30°C, 20°C and 10°C**

Different cloned *Pm* variants (pHH100) was allowed to express in *Pseudomonas putida* at varying temperature supplemented with 0.1mM m-toulate and was induced for 24 hours. The results are shown in Figure 8a, 8b, 8c. The ratio between induced and uninduced levels was calculated and identified that *Pm* variants shows higher induction capabilities compared to wild type. At 30°C it was found that pm71 shows higher induced level around thirty seven times more than induced wild type while rest variants are above twenty times higher except pm87 which shows only five times higher compared to induced wild type. Similarly higher induced values were seen at 20°C but with the same induction pattern compared to 30°C. While growing at 10°C pm85 shows higher induction level which is 164 times more compared to induced wild type. Interestingly, pm 87 shows lowest induction levels compared to other variants in three different temperatures. It was observed that induced levels were shown to be higher in lower temperature.

As, *Pseudomonas putida* is mesophilic bacteria it prefers comfortable temperature so, we can say temperature cause stress and shows leakiness in lower temperature.

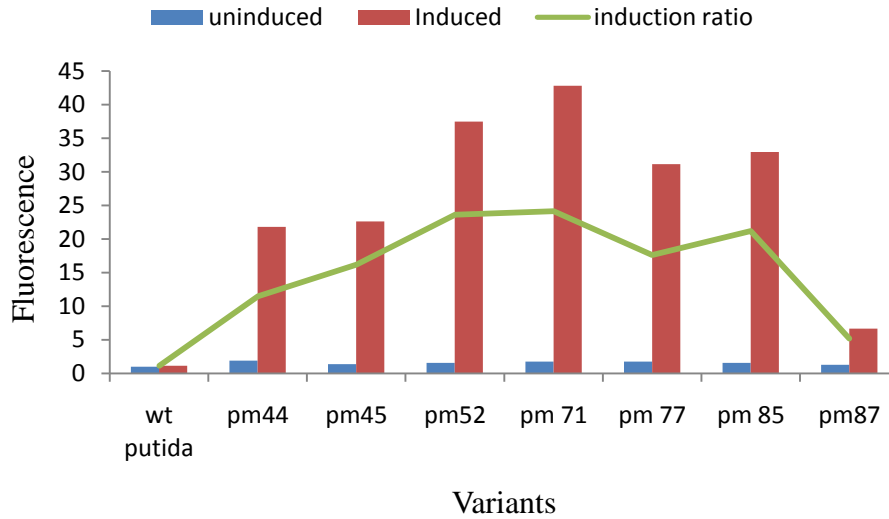


Figure 8a:-m-cherry expression levels in *Pseudomonas putida* at 30°C, 0.1mM m-toulate as inducer; induced (red bars) and uninduced(blue bars) and induction ratio (green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

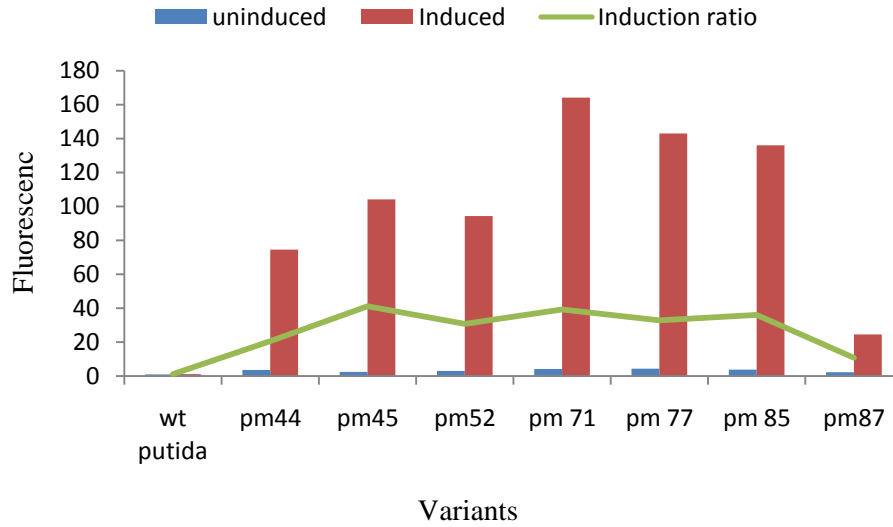


Figure 8b:-m-cherry expression levels in *Pseudomonas putida* at 20°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio(green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

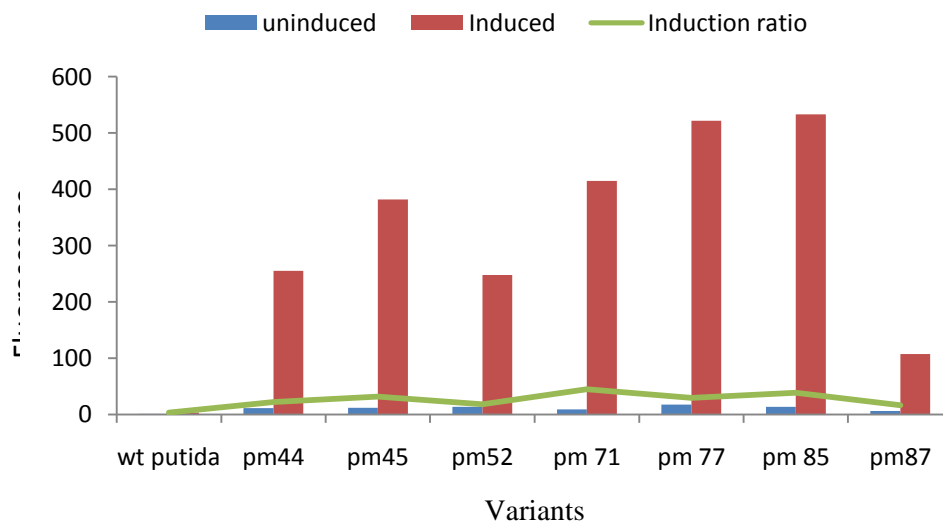


Figure 9a: -m-cherry expression levels in *Psuedomonas putida* at 10°C under induced (red bars) and uninduced (blue bars) conditions and induction ratio (green line). Wild type values are arbitrarily set to 1.

#### **8.1.1.2 6.3 Performance of *Pm* variants displaying enhanced or reduced expression levels in pSM 26 at varying temperature 30°C, 20°C and 10°C**

Similarly, different cloned variants of *Pm* were allowed to express in *Psm 26* at varying temperature. *Psm26* is psychrophilic microorganism. The experiment was carried out as described in methods section. The results are shown in Figure 9a, 9b, and 9c.

The ratio between induced and uninduced levels was calculated and it was found that identified *Pm* variants shows promising better induction capabilities compared to wild type at 30°C. While, induction was carried increasing temperature from 10°C to 30°C the induced value increases rapidly may be of leakiness caused by temperature stress as *Psm26* prefers lower temperature. The induced level was found comparatively high at 30°C, where *pm 85* shows higher induced level compared to other variants and wild type. While at temperature 20°C *pm52* and *pm71* shows slightly higher compared to wild type but other variants were found lower compared wild type whereas, at 10°C variants lose their induction level compare to wild type. Interestingly, *pm 87* shows lowest induced level compared to all other variants in three different temperature.

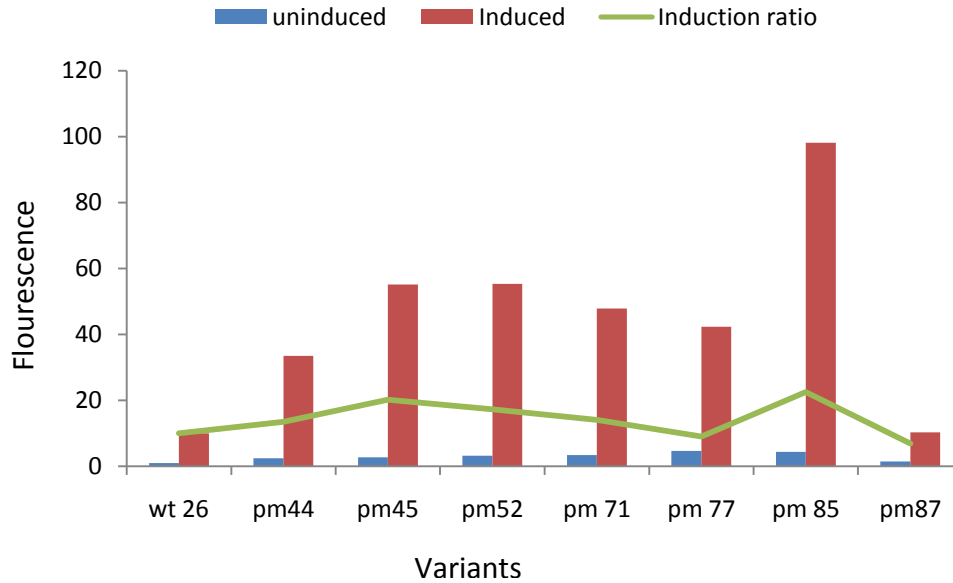


Figure 9a:-m-cherry expression levels in Psm26 at 30°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio(green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

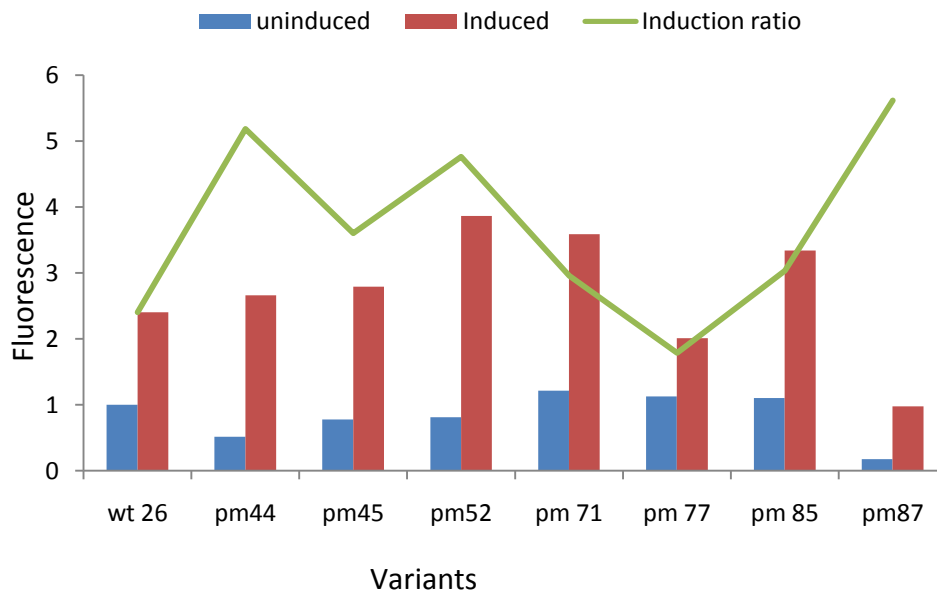


Figure 9b:-m-cherry expression levels in psm26 at 20°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio (green line). Wild type values are arbitrarily

set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

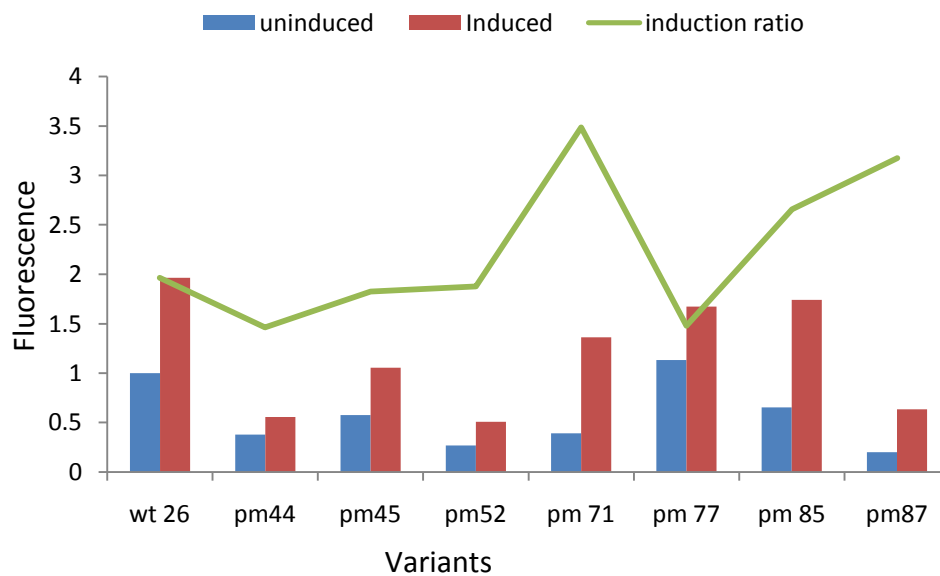


Figure 9c:-m-cherry expression levels in psm26 at 10°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio (green line).. Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

### 8.1.1.3

#### 8.1.1.4 6.4 Performance of Pm variants displaying enhanced or reduced expression levels in pSM32 at varying temperature 30°C, 20°C and 10°C

Similarly, different cloned plasmid, variants of *Pm* obtain from screening as mention above was allowed to express in *Psm 32* hosts at varying temperature. Since *Psm32* is also a psychrophilic microorganism it prefers lower temperature than higher temperature. The experimental process was carried out as described in methods section. The results are shown in Figure10a, 10b, 10c.



The ratio between induced and uninduced levels was calculated and it was found that identified *Pm* variants lose their high induction capabilities compared to wild type in pSM32. The induced *pm* 52 shows similar induction level to induced wild type while rest of the *pm* variants have reduced induced capacities compared to wild type at 30°. While at temperature 20°C *Pm*71 showed quiet higher induced level compared to wild type but rest of the variants shows reduction in induction capacities compared to wild type. But at 10°C it was found that *pm* 85, and *pm* 45 shows improved induced condition compared to wild type which shows promising candidate for further studies. It was identified that *pm*87 also shows same lowest induced level for Psm32 compared to other variants and similar nature as shown by *psm*26 where increase in temperature cause leakiness due to temperature stress.

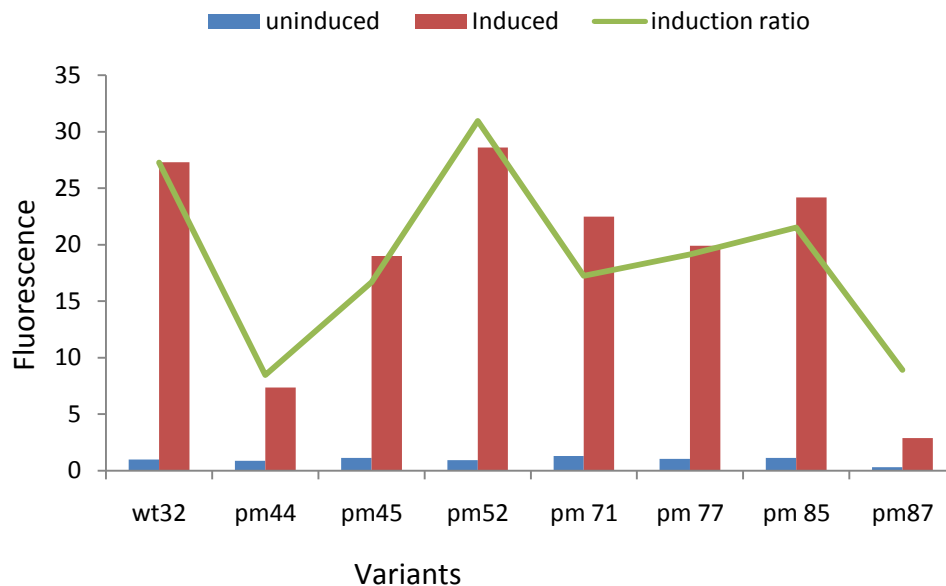


Figure 10a:-m-cherry expression levels in *psm*32 at 30°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio(green line).. Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

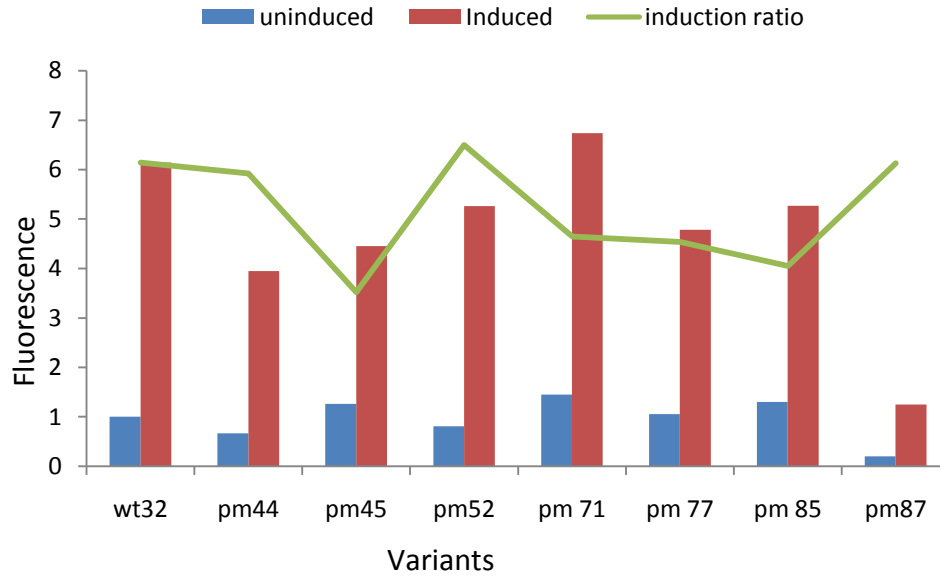


Figure 10b:-m-cherry expression levels in psm32 at 20°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio(green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

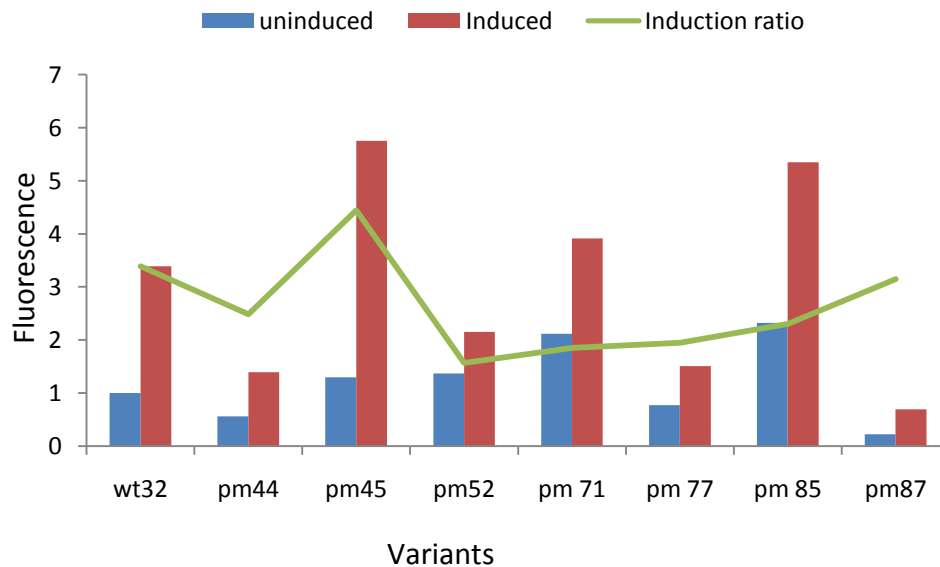


Figure 10c:-m-cherry expression levels in psm32 at 10°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio(green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

It has been previously reported that in absence of inducer Pm promoter has relatively low level of background expression. The expression system was shown to be further improved by introducing mutation into the -10 region of pm[58]. These identified variants of *pm* promoter used in this studies were previously constructed by randomly mutagenesis at 24-bp region of *Pm* in *E.coli* [57] and shows to improve in recombinant gene expression. The result presented here shows higher expression and functional especially in *Pseudomonas putida* but two psychrophilic organisms does not seems functional in contrast *pseudomonas putida*. While comparing expression pattern between two Psychrophilic organism pSM32 shows more functional compared to Psm26. Interestingly, *Pm71* seems to be more functional in these three hosts which contain deletion mutation near transcriptional start site. It was reported that particular nucleotide position was not intentionally deleted which may be generated while cloning[57].Expression level was influenced as there was mutation near transcriptional start site which influence start site selection[59]. Surprisingly it was noted that pm85 shows higher expression level in all different hosts which only contains only one point mutation. So, it is unknown that whether the ratio of point mutation will also affect expression level so in order to confirm further experiment is needed. Also, Pm45 which contain insertion mutation also shows higher expression level compared to other variants in all three different hosts. Similarly, pm87 shows lowest induction levels induction levels in all three hosts which shows tight regulation *pm* control. Also, parameter like temperature stress is also shown to be more important in expression level where *Pseudomonas putida* performs better at 30°C and pSM26 and pSM32 at 10°C and host dependency too.

**8.1.1.5 6.5 Performance of 5'-UTR variants displaying enhanced or reduced expression levels in *Pseudomonas putida* at varying temperature 30°C, 20°C and 10°C**

The 11 different cloned 5'-UTR variants were carried for the study of expression level using *Pseudomonas putida* as hosts at varying temperature i.e. 30°C, 20°C and 10°C. The results are shown in Figure 11a, 11b, 11c

The ratio between induced and uninduced levels were calculated and it was found that identified 5'-UTR variants shows higher expression levels compared to wild type. Utilising *Pseudomonas putida* as host different variants shows different expression levels at varying temperature. It was noticed that induced values increase as temperature decreases because leakiness was observed maybe stress while growing at lower temperature. Reporting at 30°C it was found that induced UTR45 shows 100 times higher induced level compared to induced wild type and UTR 77 which was reported to be lowest induced value which shows only 5 times more compare to induced wild type, likewise rest of the variants with higher induced value are listed in descending order from higher to lower UTR45>UTR84>UTR44>UTR73>UTR69>UTR46>UTR47>UTR87>UTR85>UTR88>UTR77. Uninduced value are almost similar where UTR45 and UTR 85 only shows double to wild type uninduced value. Similarly, at 20°C same characteristic and pattern was noticed as shown in 30°C but with higher induction values. While, in temperature 10°C all of uninduced and induced UTR variants show rapidly higher value compare to that 30°C and 20°C where UTR 88 shows lowest induced value compare to induced wild type.

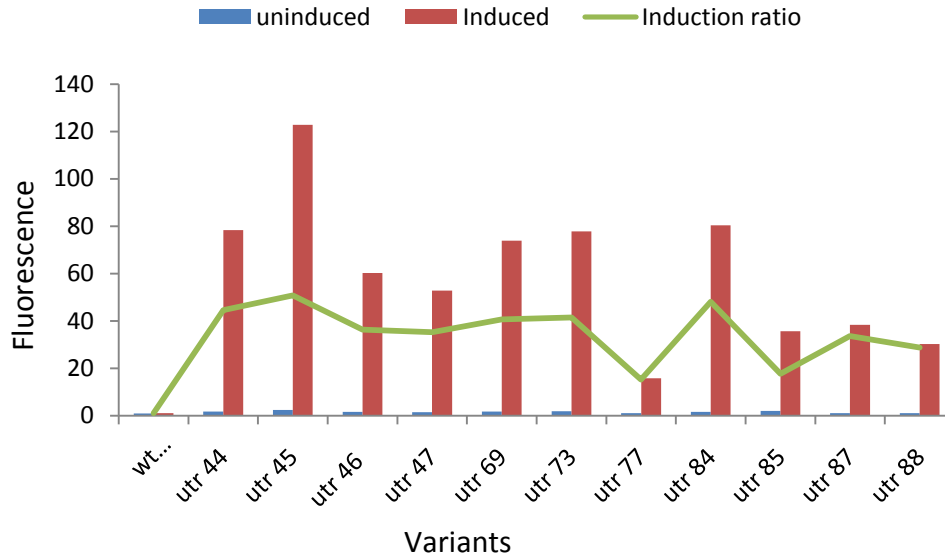


Figure 11a: -m-cherry expression levels in *Pseudomonas putida* at 30°C under induced (red bars) and uninduced (blue bars) condition and induction ratio (green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

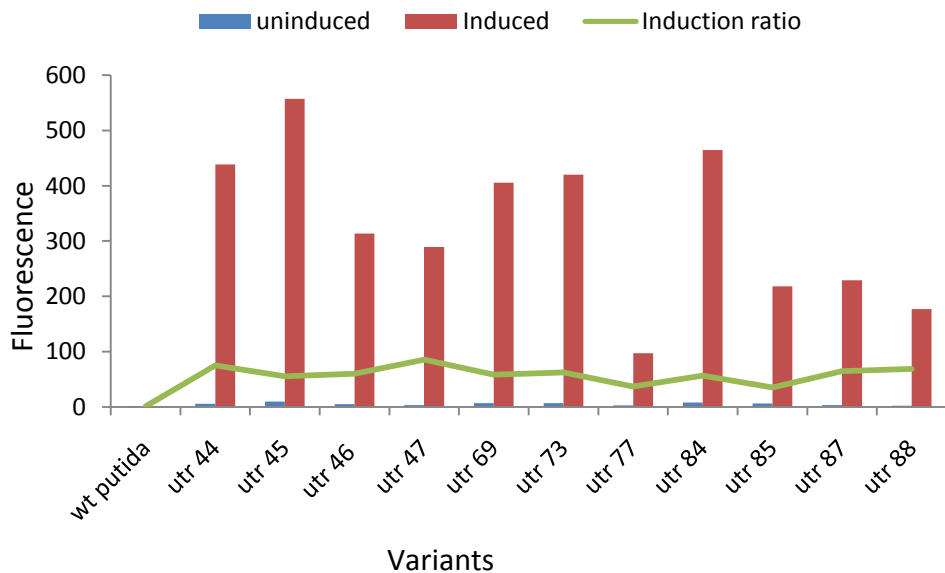


Figure 11b: -m-cherry expression levels in *Pseudomonas putida* at 20°C under induced (red bars) and uninduced (blue bars) conditions and induction ratio (green line). Wild type values are

arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

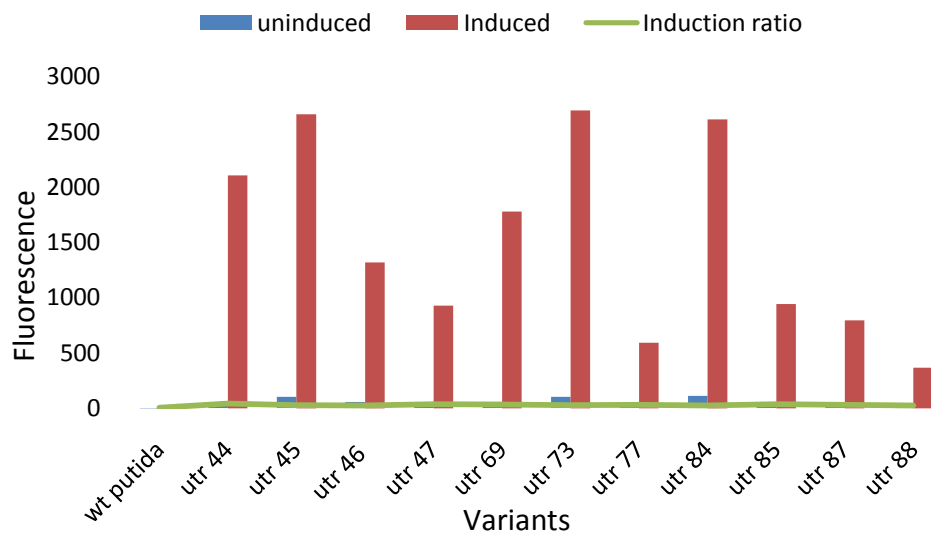


Figure 11c:-m-cherry expression levels in *Pseudomonas putida* at 10°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio (green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

### 8.1.1.6

#### 8.1.1.7 6.6 Performance of 5'-UTR variants displaying enhanced or reduced expression levels in PSM26 at varying temperature 30°C, 20°C and 10°C

Similarly, cloned variants of 5'UTR were allowed to express utilising psm26 as host at varying temperature. The induction ratio was calculated and UTR variants in PSM26 lose their induction capacities compared to wild type. The results are shown in Figure12a, 12b, 12c.

At temperature 30°C UTR 73 shows highest induction value which is twenty five times higher where UTR 45 shows the lowest induced value i.e. five times higher than induced wild type

value. In 20°C, induced values of UTR variants are almost similar to wild type value. While in 10°C induction values are shows higher compared to wild type but induction ratio were similar as wild type.

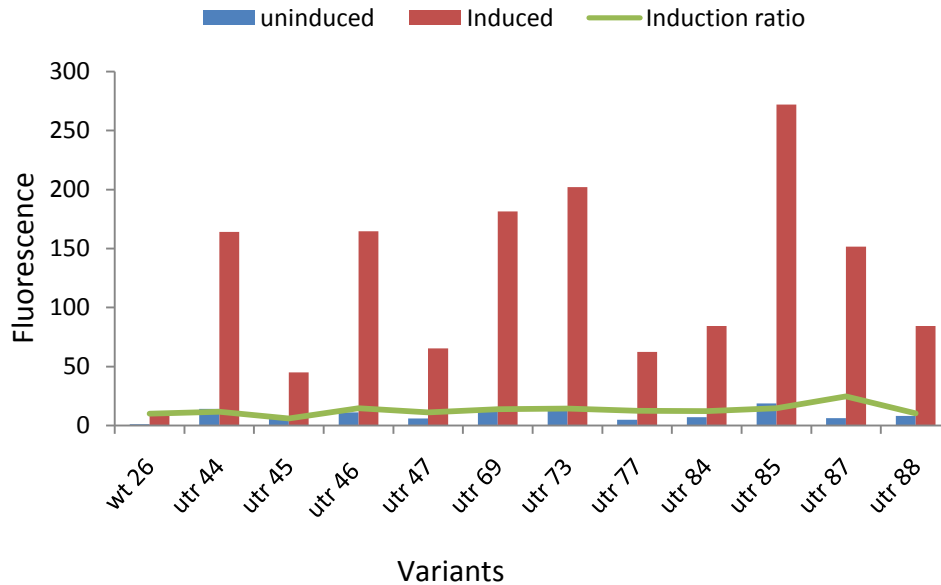
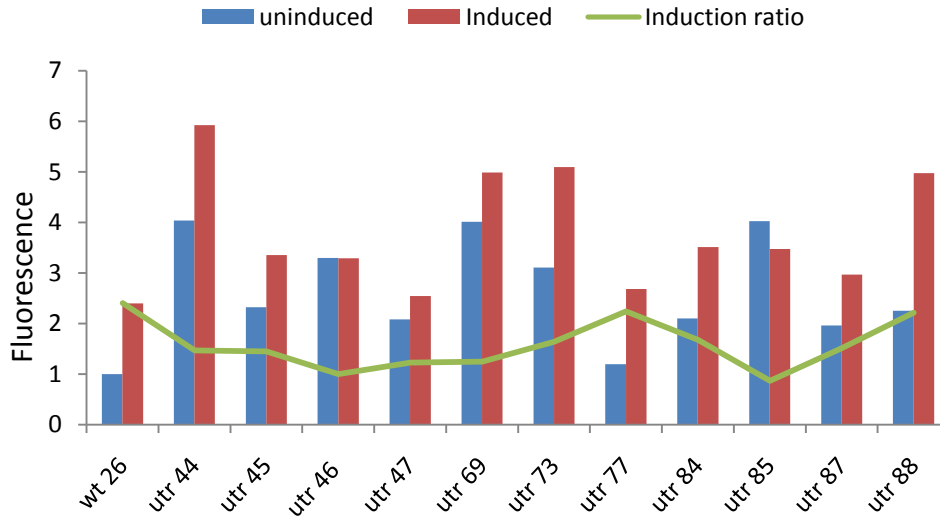


Figure 12a:-m-cherry expression levels in psm26 at 30°C under induced (red bars) and uninduced(blue bars)condition and induction ratio(green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.



Variants

Figure 12b:-m-cherry expression levels in psm26 at 20°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio(green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

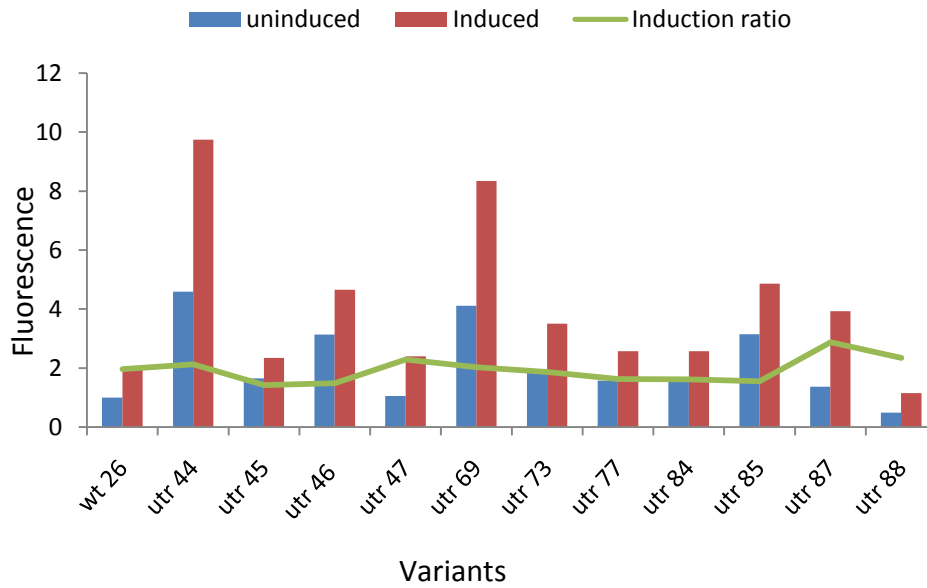




Figure 12c:-m-cherry expression levels in psm26 at 10°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio (green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

#### **8.1.1.8 6.7 Performance of 5'-UTR variants displaying enhanced or reduced expression levels in Psm32 at varying temperature 30°C, 20°C and 10°C**

The 11 different cloned variants of 5'-UTR were used for the study of expression level using Psm32 as hosts at varying temperature. Induction ratio was calculated but UTR variants in psm32 lose their induction capacities compared to wild type. The results are shown in Figure10a, 10b, 10c

It was found that at 30°C induced value of UTR87, UTR44, and UTR45 shows higher induced value compare to wild type. Similarly, UTR44, UTR73 and UTR84 shows higher induction levels compared to wild type at 20°C. There was no any significance improvement in induction levels of UTR variants at 20°C compared to wild type induced value. But at 10°C UTR85, UTR73and UTR45 shows higher induction levels compared to wild type.

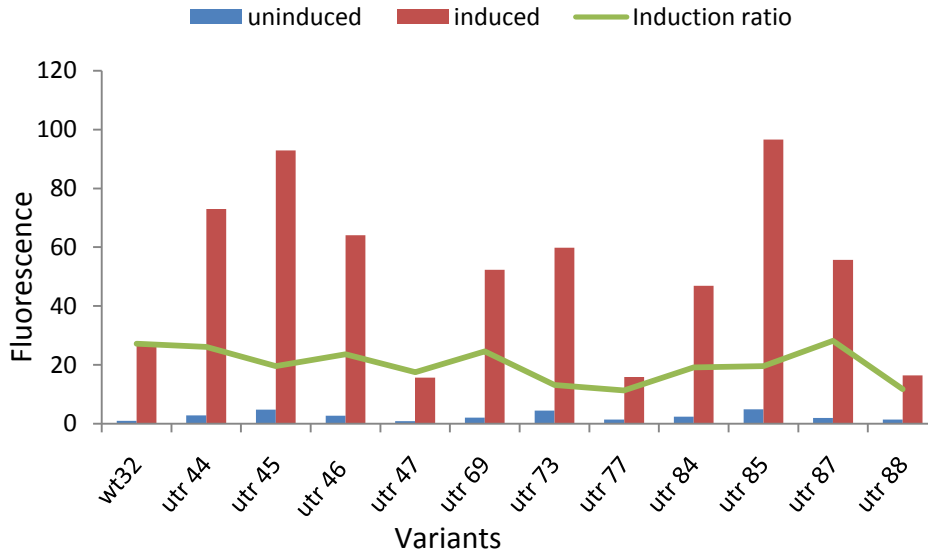


Figure 13a:-m-cherry expression levels in psm32 at 30°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio(green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

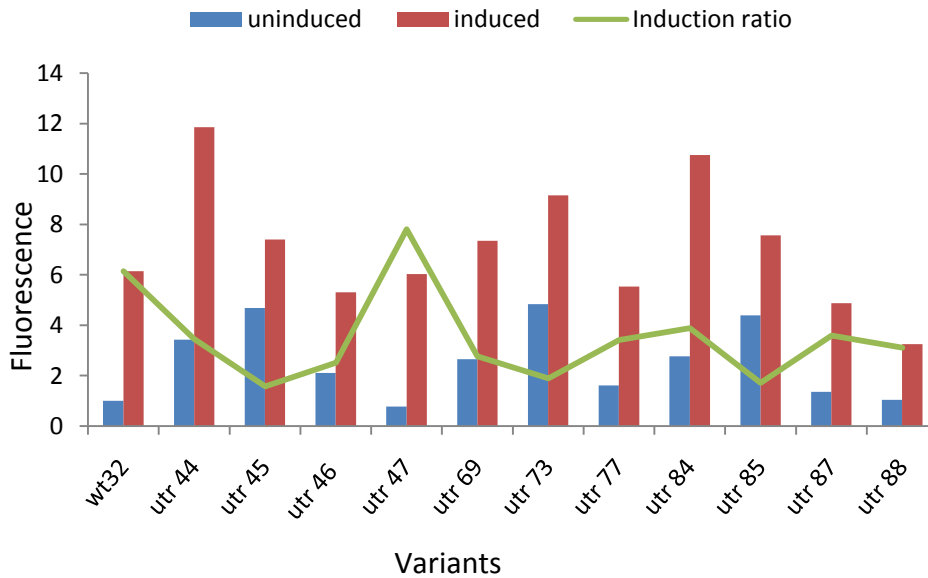


Figure 13b:-m-cherry expression levels in psm32 at 20°C under induced (red bars) and uninduced(blue bars)conditions, induction ratio(green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

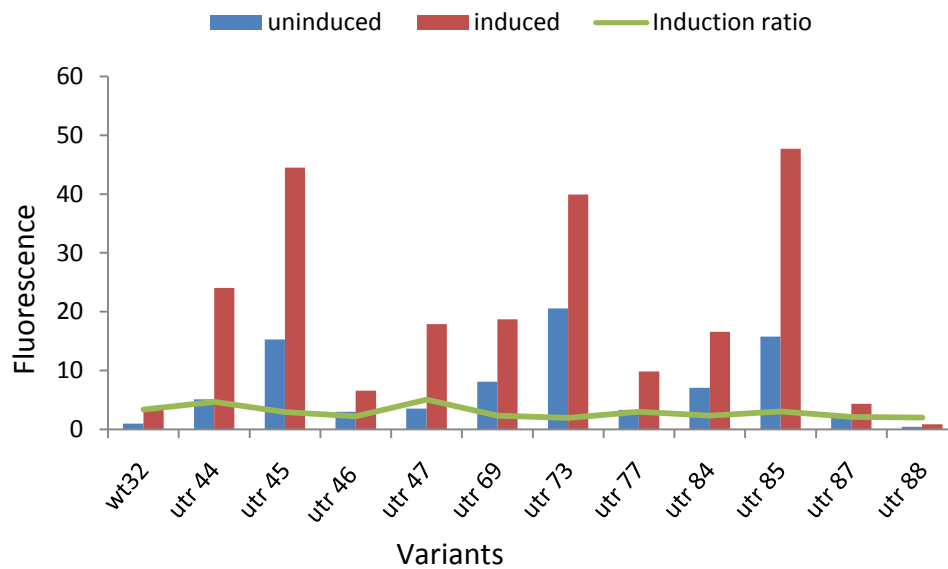


Figure 13c:-m-cherry expression levels in psm32 at 10°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio(green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

In previous studies it was identified that mutation in 5'-UTR leads to further improvement in gene expression and the identified 5'UTR variants studied in this work were previously constructed by Lale et .al, [55].It was known that translation result depends on UTR. Our result also supports that mutating the DNA region at 5'-UTR leads to improve in expression level. But our aim was to identify whether this system can be established in pseudomonas species. To our

finding it was observed that 5'-UTR leads for further improvement in *Pseudomonas putida* while other psychrophilic hosts does not shows promising improvement compared to wild type. But, it was notices that host dependency also affect expression levels. *Pseudomonas putida* performs better at 30°C in contrast to psm26 and psm32 performs better at 10°C. These psm26 and psm32 (unpublished) were provided by Rahmi. As both are psychrophilic bacteria but psm32 performs better than psm26. The most of mutated SD sequence shows reduction in expression level in all three different hosts, where it can be correlated to previous finding that mutations within SD region reduce the affinity of ribosome[1] which reduce reduced expression level..

### **6.8 Performance of combination of both Pm 5'-UTR variants displaying enhanced or reduced expression levels in *Pseudomonas putida* at varying temperature 30°C, 20°C and 10°C.**

The combination of both *pm* and 5' UTR variants can lead for further improvement than being used separately. So different clone variants (pm5'UTR) were allowed to express in *Pseudomonas putida*, at varying temperature. Combination of both variants *pm* and 5'UTR shows higher expression level compared to wild type in an order as P+U45>P+U85>P+U44>P+U87>P+U77 in both induced and uninduced condition where all of the variants shows similar expression pattern phenotype in 30°C, 20°C and 10°C (Figure:-11a, 11b, 11c).

It was found that only P+U85 (combination of both elements) shows improvement induction levels compared to elements studied separately at three different temperature. It was found that p+u85 leads to three times higher induced levels compared to those element separately. It can be conclude that combination of both elements leads for further improvement in gene expression rather than to express separately.

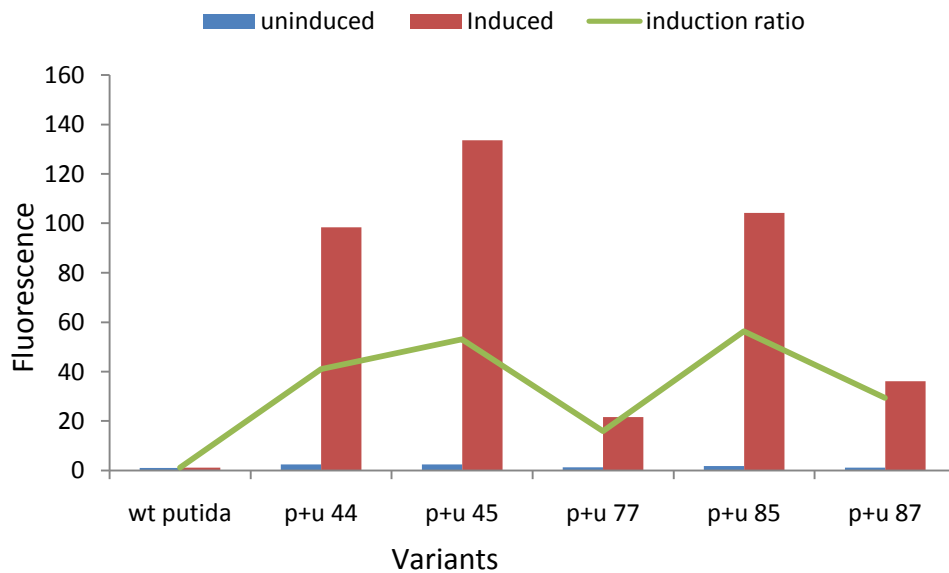


Figure 14a: -m-cherry expression levels in *Pseudomonas putida* at 30°C under induced (red bars) and uninduced (blue bars) conditions and induction ratio (green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

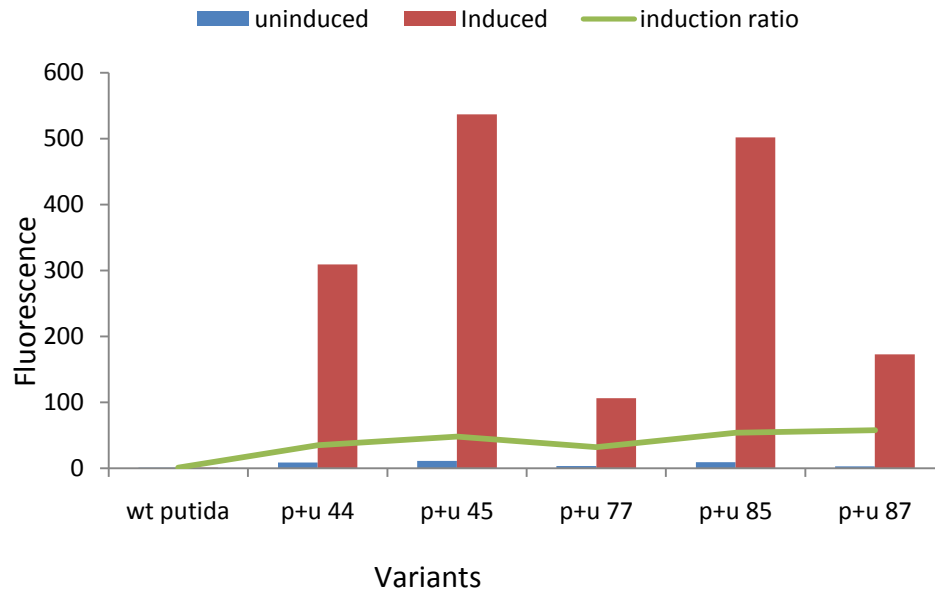


Figure 14b: m-cherry expression levels in *Pseudomonas putida* at 20°C under induced (red bars) and uninduced (blue bars) conditions and induction ratio (green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

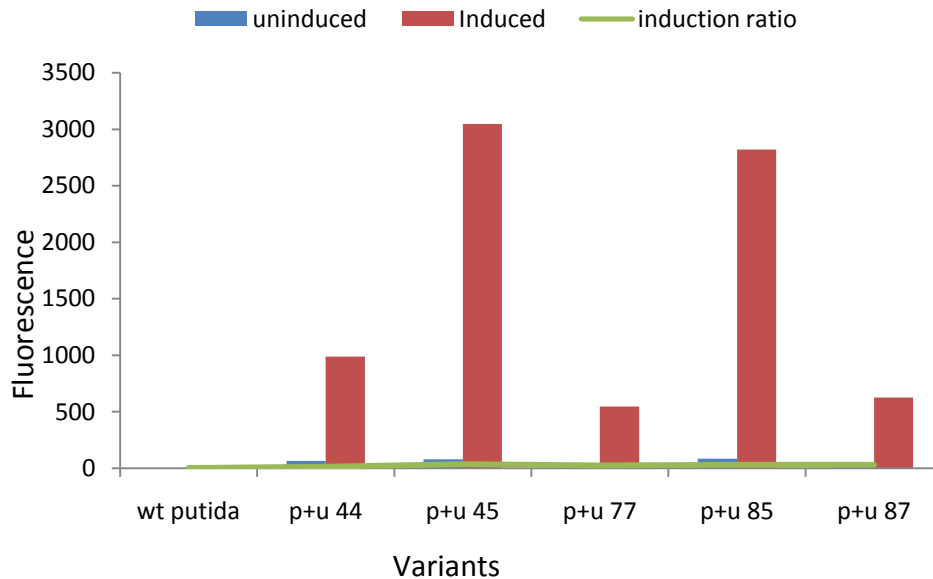


Figure 14c: -m-cherry expression levels in *Pseudomonas putida* at 10°C under induced (red bars) and uninduced (blue bars) conditions and induction ratio (green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

#### 8.1.1.9 6.9 Performance of combination of both Pm 5'-UTR variants displaying enhanced or reduced expression levels in Psm 26 at varying temperature 30°C, 20°C and 10°C.

While expressing different combination of pm 5'-UTR variants in PSM 26 it was found that all of the variants shows higher expression levels compared to wild type, where P+U45 and P+U85 gives higher induction value at 30°C and 10°C where P+U44 gives at 20°C (Figure 12a, 12b, 12c). Interestingly, it was found that P+U45 and P+U85 growing at 10°C shows further improvement in expression level than to expressing them separately. Induced P+U45 shows four times higher than induced wild type while when they are studied separately they alone show similar induced value to induced wild type. Similarly, induced P+U85 also shows four times higher value than wild type and when studied separately show similar expression to wild type.

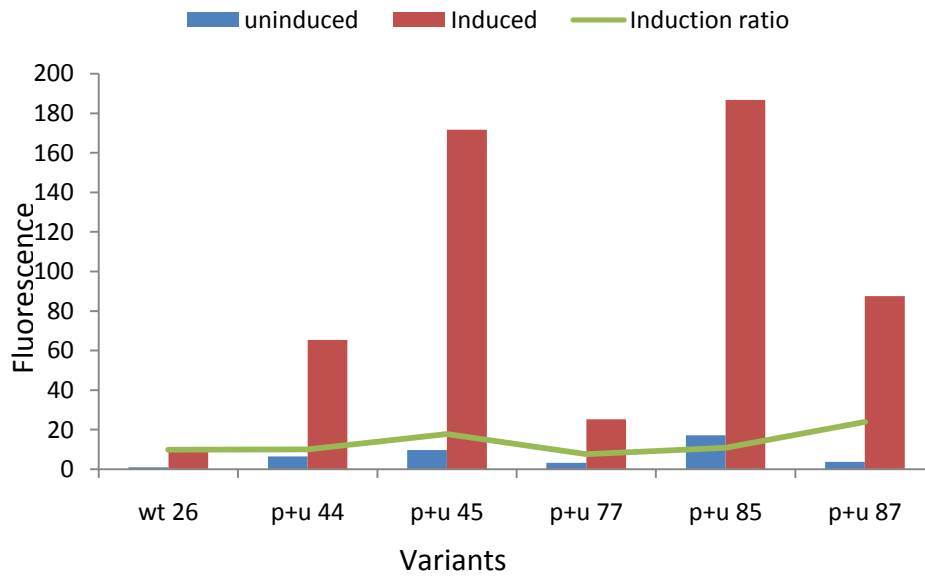


Figure 12a:-m-cherry expression levels in psm26at 30°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio (green line). Wild type values are arbitrarily set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

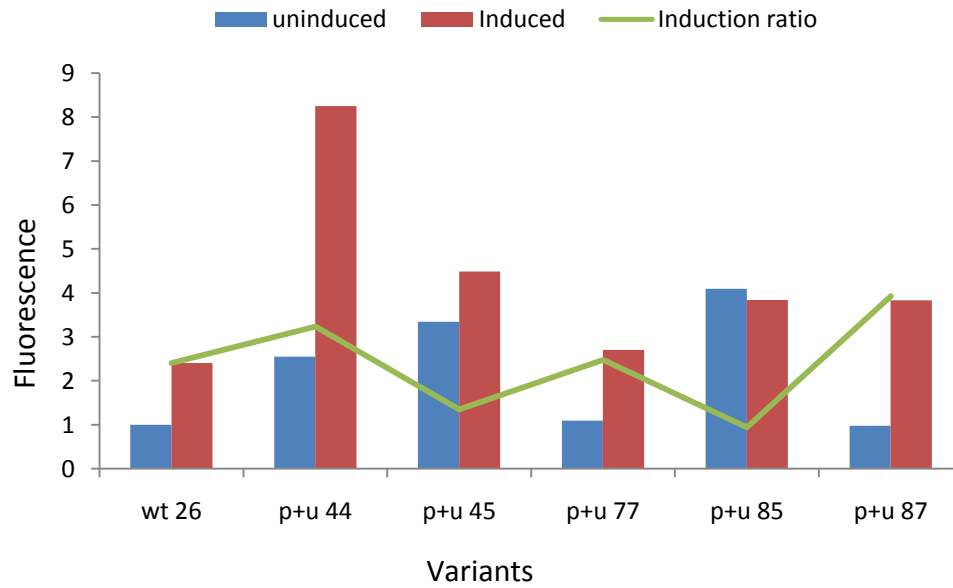




Figure 12b:-m-cherry expression levels in psm26 at 20°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio(green line). Wild type values are arbitrarily set to 1.Data represent the average of two biological replicas and induction was carried for overnight.

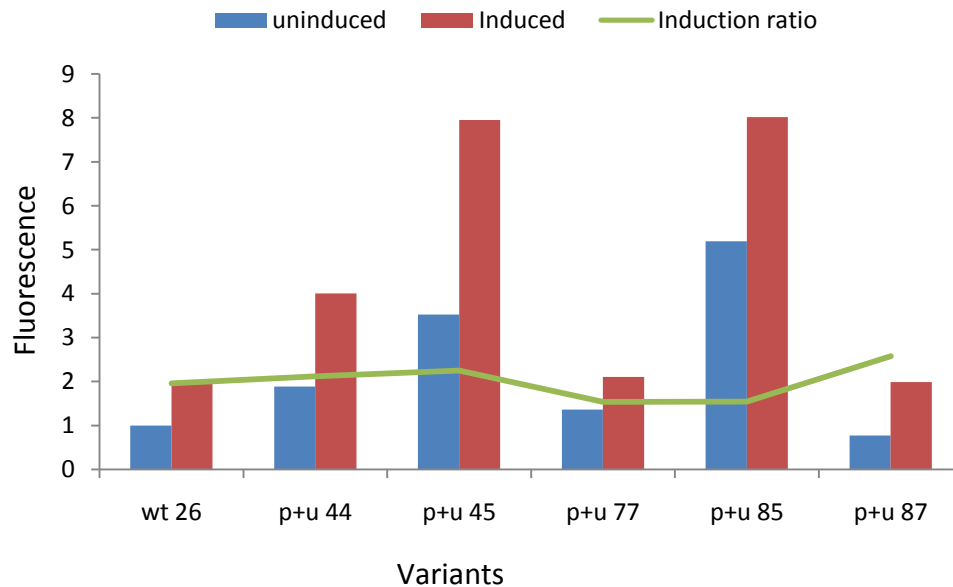


Figure 12c:-m-cherry expression levels in psm26 at 10°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio(green line). Wild type values are arbitrarily set to 1.Data represent the average of two biological replicas and induction was carried for overnight.

#### 8.1.1.10

#### 8.1.1.11 **6.10 Performance of combination of both Pm 5'-UTR variants displaying enhanced or reduced expression levels in Psm 32 at varying temperature 30°C, 20°C and 10°C.**

Similarly, different combination of variants was allowed to express using psm32 as host where the experimental procedure was carried as shown in methods section. It was found out that all of the combined pm5'UTR variants shows higher expression level compared to wild type where,

P+U44,P+U45 ,P+U85 shows relatively higher expression compared to other variants at 30°C and 20°C, while,P+U45 shows higher induction value at 10°C(Figure 13a,13b, 13c).

Surprisingly while in psm32 none of these five combined Pm5'UTR elements leads to further improved expression level in all three varying temperature. It was found UTR elements alone shows similar or more expression level compared to combination of both.

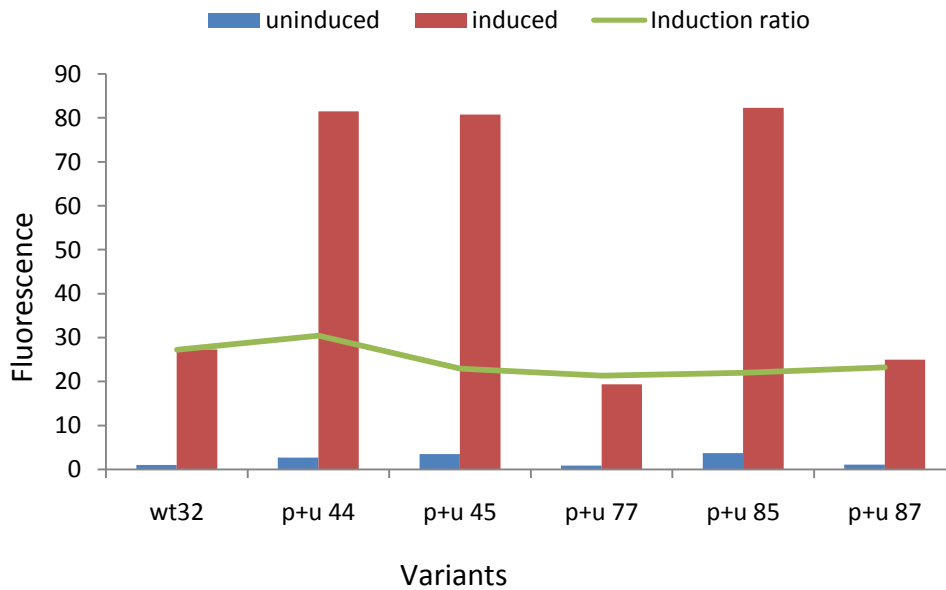


Figure 13a:-m-cherry expression levels in psm32 at 30°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio(green line). Wild type values are arbitrarily set to 1.Data represent the average of two biological replicas and induction was carried for overnight.

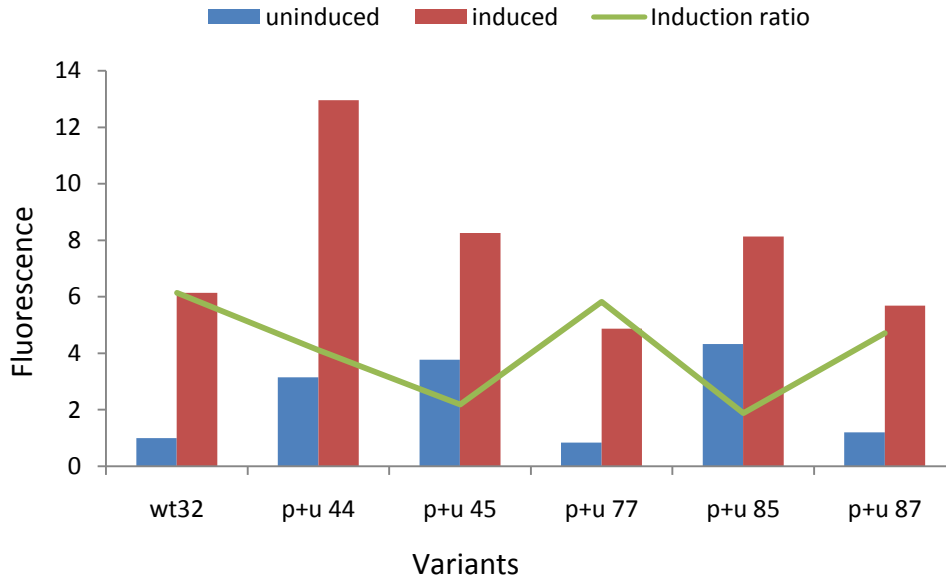


Figure 13b:-m-cherry expression levels in psm32 at 20°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio (green line). Wild type values are arbitrarily set to 1.Data represent the average of two biological replicas and induction was carried for overnight.

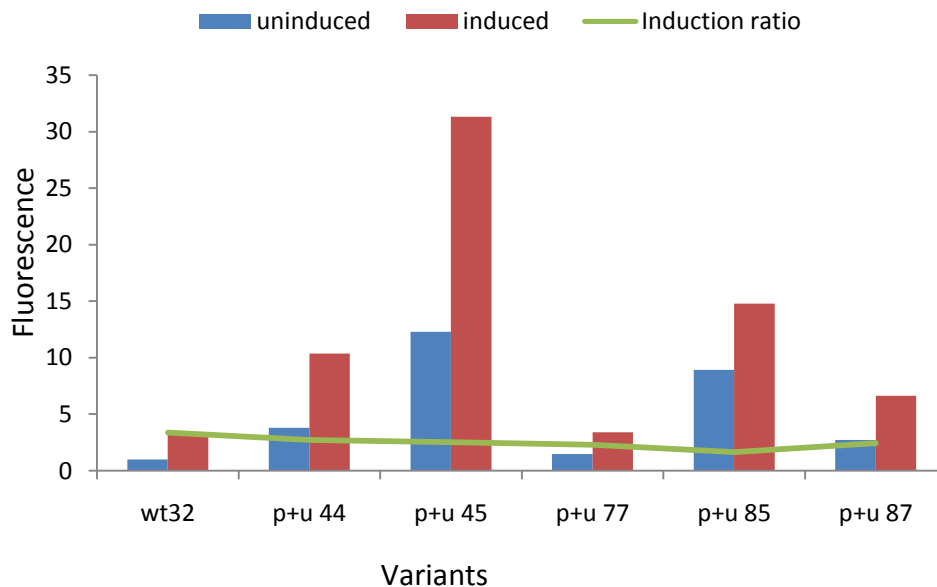


Figure 13c:-m-cherry expression levels in psm32 at 10°C under induced (red bars) and uninduced(blue bars)conditions and induction ratio(green line). Wild type values are arbitrarily

set to 1. Data represent the average of two biological replicas and induction was carried for overnight.

In this studies we have choose to combine both pm 5'UTR variants to check whether it leads for further improvement in gene expression in *Pseudomonas* hosts. It has been previously noticed that combination of both elements would lead for the improvement[1] Also it was identified in this study that on combination of these two regulatory element leads for further improvement. P+U85 in *Pseudomonas putida* and P+U45;P+U85 in psm 26 leads for further improvement. Interestingly, the promoter that contain only one point mutation and another promoter sequence with insertion mutation and combination with 5'UTR sequence without SD mutation leads for further improvement. But unfortunately, this was not observed in Psm32 means we can say expression system rely on host dependency.

#### **8.1.1.12 Comparing of expression level of pm 5'-UTR identified in different hosts.**

Different variants of pm and 5'-UTR indentified were allowed to express on all three different hosts. It was found that *Pseudomonas putida* shows much higher induced level and induction ratio compared to psychrophilic psm26 and psm32 bacteria at different temperature. But surprisingly, psm26 shows quiet higher induction level at 30°C than *pseudonamas putida* and psm32 may be of leakiness. On the other hand comparing both psychrophilic bacteria Psm32 shows higher induced levels than psm26 at 20°C and 10°C. It was find out that *Pseudomonas putida* perform better at 30°C while other performs better at 10°C and 20°C. Though different variants have been picked from different hosts it was found that pm 5'-UTR shows host independent performanance.

<i>Pm</i> variants	5'UTR variants	<i>Pm</i> 5'- UTR variants	Hosts
Pm44 Pm45 Pm52	UTR 44 UTR45 UTR46 UTR47	P+U44 P+U45	<i>Pseudomonas putida</i>
Pm71 Pm77	UTR69 UTR73 UTR77	P+U77	<i>Psm26</i>
Pm85 Pm87 Pm88	UTR84 UTR85 UTR87 UTR88	P+U85 P+U87	<i>Psm32</i>

Table1:- Identified *pm* 5'-*UTR* variants and hosts.

## **8.2 7.Result and discussion ( Synechocystis)**

### **8.3 7.1 Conjugative transfer of pHH100 into Synechosystis PCC6803**

pHH100 which is broad host range plasmid was used as a transconjugation vector. Conjugation procedure was carried out using *E.coli S17.1* as donor cell, described in the methods section . Cells were plated on BG11 plates with kamanycin 25µg/ml and was incubated in 30°C with continues supply of light. Surprisingly, transconjugants were observed after 6 days of incubation. So, in order to confirm transfer, further analysis was carried out by plating these transconjugants in BG11 plates supplement with kanamycin and incubated at 30°C with continuously supply of light but observing for several days it was noticed lack of kam<sup>r</sup> colonies. This plasmid use as transconjugants cannot replicate and maintain autonomous replicon in Synechosystis PCC6803.

## **8.4 Future perspectives**

The tested psychrophilic hosts are promising alternatives for *E.coli* to accomplish protein expression at low temperature. In the future it would be valuable to evaluate protein expression levels in identified stains showing high gene expression.

Although initial studies indicates that the tested plasmid cannot be transferred or replicated in *Synechocystis* 6803, it will be interesting to check whether Xyls/*Pm* system can be implemented using another vector system.

## 8.5 Conclusion

The aim of this study was to screen and identify potential *pm* and 5'-UTR variants and to check the expression profile in different *Pseudomonas* hosts. The result show that *pm* and 5'-UTR system seems to be functional in *Pseudomonas putida*, pSM26 and pSM32. Where *Pseudomonas putida* KT2440 shows higher expression compare to psychrophilic *Pseudomonas* strains (pSM26 and pSM32) bacteria. Combination of both *Pm* variants and 5'-UTR variants that yield high expression leads to a further increase in protein expression in *Pseudomonas putida* KT2440 and pSM26. In contrast, pSM32 show higher expression when *pm*, 5'UTR elements are employed separately at 10°C compared to pSM26. Temperature also plays major role in protein expression level. The promoter appears to show leakiness in all three hosts where *Pseudomonas putida* KT2440 shows at lower temperature i.e. 10°C and psychrophilic *Pseudomonas* strains (pSM26 and pSM32) at higher temperature i.e. 30°C.

Attempts to transfer RK2-based broad host range plasmid pHH100 form *E.coli* to *Synechocystis* PCC6803 through conjugation was not seen. The reason for this could be that the plasmid is not transferred or cannot replicate in *Synechocystis*.

9



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