Isolation and Characterization of str Promoter from Bacteria *Escherichia coli* DH5α using Reporter Gene AmilCP (*Acropora millepora*)

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**Abstract.** In heat stress conditions, *E. coli* will produce EF-Tu protein abundantly under the regulation of str promoter. The str promoter from *E. coli* is known to have sequences that are recognized by two sigma factors (σ⁷₀ and σ⁵). The aim of this research was to isolate and characterize str promoter from *E. coli* DH5α using reporter gene AmilCP. The str promoter from *E. coli* DH5α (289 bp) had been isolated by Colony PCR using specific primers. Bioinformatics analysis of str promoter showed high conserved areas (100%) for some kinds of *E. coli* strains. The str promoter was constructed in pSB1C3 vector by modifying OEPC to regulate reporter gene AmilCP. Qualitatively, the ability of str promoter constructs in regulating AmilCP gene was observed as purple-blue colonies of *E. coli*. Quantitative analysis was performed by measuring cell extract of *E. coli* at a wavelength of 588 nm after treated at different temperatures (4, 24, 37, 45, 50, 55 and 60 °C) for 1 hour. The ability of str promoter to express reporter gene AmilCP was compared to one powerful promoter which is the lacUV5 promoter. The results show that both promoters are functional promoters and can be induced by temperature (50 °C).

1. **Introduction**

Stress is an external factor that can affect living things. Naturally, bacteria have stress sensing systems and the ability to fight stress conditions so they are able to survive from environmental changes or extreme conditions [1]. In some bacteria, there is a stress control system by a single master regulator. *E. coli* and some of bacteria in the class of gammaproteobacteria are controlled by the master regulator σ⁵ [2].

Temperature is an abiotic factor that can influence bacterial growth. When the temperature reaches a threshold at a certain time, bacteria show their stress to inhibit growth and metabolism process. Furthermore, high temperature directly causes cellular damage like disrupt membrane stability, denaturation, and aggregation of cellular proteins [3].

During heat stress (50 °C), there is an EF-Tu protein in *E. coli* which is overexpressed under promoter regulation [4]. The EF-Tu protein acts as a chaperone molecule that can bind to the hydrophobic residues of open proteins, thus it prevents the formation of aggregation and restoring conformation of the original protein when conditions return to normal [5].

The ability of str promoters to express EF-Tu proteins abundantly during heat stress tracts researcher’s attention. Presently, around 50 str promoters have been successfully sequenced from
several different species such as *E. coli* [6], *B. Stearothermophilus* [7], *S. platensis* [8], *C. trachomatic* [9], and *S. typhimurium* [10], but only a few of these promoters have been functionally characterized in transcription processes and products. Promoter is a sequence of DNA that can interact with transcription factors and RNA polymerase, therefore, the sequence can control gene expression [11]. Promoters have an important role in controlling the production of recombinant proteins. By controlling the initiation stage of the transcription process, cells can regulate the amount of protein to be produced and how fast the expression process takes place.

High temperature regulated promoters have been successfully characterized and patented on various HSP promoters (US5447858A; US5521084A; US6852511B2; US5858740) and promoters pL / pR (EP0173280A1; EP0177228AA; US5085740) (US5858740) and promoters pL / pR (EP0173280A1; EP0177228AA; US5085740) (US5085740) [12]. The expression system under promoter regulation has been successfully used in the bioreactor to produce recombinant proteins. This is due to easy regulation and strong promoter character without the addition of certain expensive and toxic chemical inducers [13].

The principle of the high temperature-regulated promoter in step with the conditions of the bioreactor which brings in heat transfer during the production process [14]. This condition is very suitable for producing bacterial-based therapeutic recombinant proteins in bioreactor. New research in finding a high-temperature promoter is a breakthrough in the bioprocess field and adds the list of functional promoters in the production of recombinant proteins.

2. Material and Methods

2.1 Identification of *E. coli* DH5α.

*E. coli* DH5α bacteria used to study the strains uses a method 16S rRNA DNA. Amplification of 16S rRNA gene was produced using Dream Taq Green DNA Polymerase. DNA sequences are analyzed using the BLASTN program and the MEGA 6.06 software.

2.2 Isolation of str promoter from *E. coli* DH5α.

The forward and reverse primer are designed for amplification of str promoter. Purification of DNA sequences by GeneAid gel/PCR DNA fragments Extraction kit (USA). The str promoter fragments are ligated to pJET 1.2 vector, and the results are transferred to *E. coli* competent cells by a transformation procedure.

2.3 The construction of AmilCP gene to str promoter.

Overlap Extension PCR Cloning (OEPC) consists of two stages: overlap-extension PCR and PCR cloning. The overlap-extension PCR functions are to produce the str promoter (mega primer). While PCR cloning is used to enter the str promoter into the pSB1C3 vector (Bba_K592025).

2.4 The construction of AmilCP gene to lacUV5 promoter.

The AmilCP gene was amplified by KAPA Hifi HotStart PCR Kit (USA). The purification products were ligated to the pJET1.2 vector.

2.5 Characterization of str promoter from *E. coli* DH5α.

The expression of AmilCP gene was observed under the regulation of both str and lacUV5 promoter by a qualitative and quantitative method. Qualitatively, the expression of the AmilCP gene showed a colony with a purple-blue color. Quantitative analysis was performed by measuring the cell extract of *E. coli* at a wavelength of 588 nm. All process was treated at different temperatures (4, 24, 37, 45, 50, 55 and 60 °C) for 1 hour.
3. Results and Discussions

3.1 Identification and Isolation of str promoter from *E. coli* DH5α

Isolation of str promoter from *E. coli* DH5α was carried out by the Colony PCR method using specific primers. The forward and reverse primers for amplification are based on conserved sequences of the promoter region [6] on all strains of *E. coli* bacteria from the GenBank sequence data. The str promoter successfully isolated with primers which recognized sequences in the upstream of the str operon region around 289 bp from the S12 gene (Figure 1).

![Figure 1. Gel electrophoresis of amplification products of the str promoter. L: 1 kb DNA ladder, -ve: negative control, Ps: str promoter from *E. coli* DH5α (289 bp).](image)

The results of BLAST str promoter sequence showed a 100% homology level, 100% query coverage and E-value 0 for several strains of *E. coli*. The phylogenetic tree of the str promoter sequence was built using the neighbor-joining method using MEGA 6.06 software.

![Figure 2. Phylogenetic tree of str promoter sequence of *E. coli* using the neighbor-joining method.](image)

The neighbor-joining method is a phylogenetic tree reconstruction method by calculating the distance from each branch of the phylogenetic tree. At each stage, the two closest nodes in the phylogenetic tree are defined as neighbors. Neighbors are pairs of OTU's (Operational Taxonomic Units) or can be assumed to be leaves of a phylogenetic tree that have one node that connects the two [15]. Based on the phylogenetic tree construction (Figure 2), the identification of str promoter DNA barcode is well done. The *E. coli* sample used shows a close relationship with several strains of *E. coli*. 
3.2 The construction of AmilCP gene to str and lacUV5 promoter.

The construction of the str promoter assembled with Bba_K592025 part (AmilCP reporting gene) using a technique of modified PCL Cloning Overlap Extension (OEPC). The process consists of two stages: overlap-extension PCR and PCR cloning. The overlap-extension PCR functions are to produce the str promoter (mega primer) that has a homologous tail sequence at the 5' and 3' ends of the pSB1C3 vector. While PCR cloning is used to enter the results of the megaprimer (str promoter) into the pSB1C3 vector (Bba_K592025) containing the AmilCP reporting gene [16].

The PCR cloning process successfully made based on the comparison of concentrations between the mega primer and the template (100:1) [17]. The modifications are used to improve the efficiency of primary overlap-extension PCR and to increase the mega primer concentration, so the success of PCR cloning is high. The results of PCR cloning were given the DpnI enzyme (0.25U). The function of the DpnI enzyme is to degrade the initial plasmid template that the str promoter cannot successfully insert because it has been methylated by E. coli. The expression of the AmilCP gene under the str promoter regulation showed a colony with a purple-blue color (Figure 3).

![Figure 3](image)

**Figure 3.** The success of the str promoter constructs to express the AmilCP gene showed a purple-blue colony (LB agar + chloramphenicol, 170 ppm). A & B colony compared to C (Bba_K592025 part).

The AmilCP gene activity was compared under the str promoter and the lacUV5 promoter. The AmilCP gene constructed into pJET1.2 vector under the lacUV5 promoter regulation. The results of the AmilCP gene amplification can be seen in figure 4.

![Figure 4](image)

**Figure 4.** Gel electrophoresis of amplification products of the AmilCP gene. L: 1 kb DNA ladder, -ve: negative control, As: AmilCP gene from Bba_K592025 part (700 bp).

3.3 Characterization of str and lacUV5 promoter from E. coli DH5α.

The ability of the str promoter and the lacUV5 promoters in expressing the AmilCP gene is compared qualitatively. AmilCP gene expression under the lacUV5 promoter showed stronger purple-blue color than a colony under the str promoter (Figure 5). This qualitative result is supported by the result of E. coli cell extract at different temperature treatments for 1 hour (Figure 6).
Figure 5. *E. coli* expresses the AmilCP gene showed a purple-blue colony under the lacUV5 promoter (A) and the str promoter (B). A & B colony compared to C (Bba_K592025 part).

Quantitative analysis was counted by viability assay and cell extracts absorption after the culture was given a different temperature treatment for 1 hour. Based on figure 6, it was found that the AmilCP protein cell extract from the lacUV5 promoter construct showed higher results (0.596) compared to the AmilCP protein cell extract from the str promoter construct (0.556). These results show a qualitatively similar to the AmilCP gene expression in figure 5.

Figure 6. *E. coli* cell extract contains the AmilCP protein showed a purple-blue color under the lacUV5 promoter regulation (A) and the str promoter regulation (B) at different temperature treatment for 1 hour. AmilCP protein absorption is highest at 588 nm.

The AmilCP protein cell extract is produced excessively at 45 °C. The temperature of 45 °C is not *E. coli* optimum temperature life because *E. coli* a type of mesophyll bacteria (25 ° - 40 °C). This condition that the expression of recombinant proteins in *E. coli* at high temperatures (45 °C). Testing the viability assay of each cell culture needs to be used as a parameter to see the ability of the expression results of the AmilCP protein per colony-forming unit (CFU).

Growth of *E. coli* shows an increase in the number of cells in the temperature range of 4°C - 45°C in the incubation period for 1 hour. Whereas at high temperatures (50 °C, 55 °C and 60 °C) shows a decrease in the number of *E. coli* cells (death) because these temperatures outside the tolerant threshold of life for mesophyll bacteria. High temperatures do not only inhibit bacterial growth and their metabolism, but also cause cellular damage includes denaturation and aggregation of cellular
proteins and disrupt membrane stability [18]. The result of AmiCP/CFU protein absorption from each sample showed in figure 7.

Figure 7. The AmiCP/CFU protein absorption from each sample with different temperature treatments (4\(^0\), 24\(^0\), 37\(^0\), 45\(^0\), 50\(^0\), 55\(^0\) and 60\(^0\)) for 1 hour. S: culture without temperature treatment.

The results of AmiCP/CFU protein absorption were obtained by dividing the results of AmiCP protein expression with the amount of viability of E. coli cells after incubation for 1 hour. Specifically for samples treated at 50\(^0\), 55\(^0\), and 60\(^0\), the number of cells used is the number of cells in the control cell culture before being treated (S). This is caused by the number of E. coli cells that has decreased during the incubation process. This condition causes the highest AmiCP protein expression results shown in the treatment of E.coli cell cultures at 50\(^0\)C.

4. Conclusion
The results showed that the str promoter has been successfull isolated (289 bp) from E. coli DH5\(\alpha\) and is constructed into a Bba_K592025 part (iGEM11_Uppsala_Sweden) containing AmiCP reporting gene with OEPC modification technique. The str promoter and lacUV5 promoter are functional promoters and can be induced by temperature. Results of the expression of the AmiCP gene showed the highest absorbance value (588 nm)/Cfu at 50\(^0\)C at both the str promoter (0.548) and the lacUV5 promoter (0.626).

5. References
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