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Review

Promoter engineering for the recombinant protein production in prokaryotic systems

Ozgun Firat Duzenli[1], Sezer Okay[2,*]

[1] Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey
[2] Department of Vaccine Technology, Vaccine Institute, Hacettepe University, Ankara, Turkey

* **Correspondence:** Email: sezerokay@hacettepe.edu.tr; Tel: +903123052030; Fax: +903123053493.

Abstract: Recombinant proteins have an economical value with their utilization in many areas from food industry to pharmaceutical and chemical industry. Therefore, it is of great importance to establish optimum production systems for the proteins of interest. One of the critical steps in protein production is regulation of the gene expression. Promoters are among the key regulatory elements which can directly control the level of recombinant gene expression in a host cell. Thus, a suitable promoter is required for optimum gene expression. Promoter engineering is an innovative approach to find out the best promoter system for the expression of recombinant genes, which influences the overproduction of proteins of interest. In this review, some of the bacterial hosts highly used in recombinant protein production were discussed. Next, the importance of promoters in recombinant gene expression, and promoter engineering for enhanced protein production were described. Utilization of double promoter systems was highlighted as one of the successful techniques in overproduction of recombinant proteins. Increment in the variety and availability of the novel methodologies especially in the synthetic biology is expected to increase the quality and the quantity of recombinant proteins with an economical value.

Keywords: double promoter; gene expression; genetic engineering; promoter engineering; recombinant DNA; recombinant protein

1. Introduction

Recombinant proteins have been produced for over 30 years in different quantities from gram to kilogram, and prices are from tens to billions of dollars. Usage ranges from food industry to pharmaceutical and chemical industry, as well as for research purposes [1]. Recombinant proteins have great market value rising day by day, irrespective of their intended use. Therefore, it is critical to establish a production system for the proteins of interest as possible as in a high-yield and cost-effective manner. However, many parameters affect yields and costs of the proteins to be produced. In order to achieve an optimal production system, the following issues should be considered: (i) selection of host organism used as synthesis machinery for the protein of interest, (ii) characteristics of genetic elements, such as promoters or terminators, and expression plasmid chosen for heterologous production of the protein of interest, (iii) providing an appropriate growth and production medium for the host cell, (iv) ensuring the protein of interest-host cell compatibility, (v) establishing an effective downstream purification strategy. The desired success underlies the proper combination of these factors [2]. Strategies such as making modifications on expression conditions, expression vectors [3] or development of novel stable plasmid systems [4] can lead to applicable and cost-effective platforms for the production of high yields of recombinant proteins.

To date, various eukaryotic and prokaryotic production hosts were used for recombinant protein production in small or large scale, for commercial or research purposes, for industrial or therapeutic reasons. Novel strategies such as utilization of different expression hosts, either eukaryotic or prokaryotic, development of novel recombinant strains, optimization of culture conditions, and modification of expression plasmid elements are used for optimal production of recombinant proteins. In this review, prokaryotic hosts used highly for the recombinant protein production are mentioned briefly. Then, the importance of promoters, special elements of an expression plasmid and their effect on the production of recombinant proteins in prokaryotic systems are described. Lastly, the strategies based on coupling of promoters for enhancing production efficiency of prokaryotic hosts are discussed.

2. Common prokaryotic hosts used in the production of recombinant proteins

Although various hosts are used for the production of recombinant proteins, some of the bacterial species are used more commonly. Each bacterial species has its own advantages and disadvantages (Table 1). Therefore, selection of the bacterial host mainly depends on the purpose and the methodology to be used.

Table 1. Advantages and disadvantages of common prokaryotic host cells used for the production of recombinant proteins.

Host	Advantages	Disadvantages
Streptomyces spp.	• Simplification of downstream process due to efficient secretion system • Effective secretion profile for prokaryotic-based proteins • Relatively low level of endogenous extracellular proteolytic activity depending on strain	• Low production levels • Large-scale fermentation problems due to mycelial lifestyle • Hampered production of eukaryotic-based protein
Bacillus spp.	• Easy and inexpensive cultivation at high cell densities • Generally Recognized as Safe (GRAS) organism • High secretory capacity • Easy adaptation to growth media	• Lack of suitable expression vectors • Plasmid instability • Presence of proteases • Occurrence of malfolded proteins
Lactococcus lactis	• Beneficial structures of lactic acid bacteria to human health • Applicability as oral vectors for protein delivery • GRAS organism	• Low expression levels • Similar bottlenecks in protein production and secretion as *Bacillus* spp.
Escherichia coli	• Fast proliferation and high expression levels • Easy, quick and cheap processing and scale-up • Broad knowledge about physicochemical properties and genome • Presence of numerous genetically manipulated strains for recombinant protein production	• Inefficient export of heterelogous proteins because of outer membrane • Inclusion body formation • Presence of endotoxins
Corynebacterium glutamicum	• GRAS organism • Lack of endotoxins • Ability to secrete heterologous proteins as properly folded and functional • Low extracellular hydrolytic enzyme activity	• In some cases, lower growth rates and transformation efficiency • Fewer available expression systems, • Lower yields of recombinant proteins • Relatively expensive culture costs

2.1. Streptomyces *spp.*

Streptomycetes are gram-positive bacteria and mostly live in soils containing plant and animal residues in abundance. Streptomycetes secrete large number of secondary metabolites including proteins which are economically valuable and have wide range of applications in pharmacy, food and textile industries. Many of these secondary metabolites are known as antibiotics. For example, *Streptomyces aureofaciensis* produces tetracycline [5], *S. clavuligerus* produces cephalosporin [6], *S. fradiae* produces neomycin [7], *S. griseus* produces streptomycin [8] and *S. venezuelae* produces chloramphenicol [9]. Secondary metabolites from *Streptomyces* spp. and their antibiotic effects are beyond the scope of this review and well-reviewed by the others [10–12]. Among the other *Streptomyces* spp., *S. lividans* is predominantly chosen as host for recombinant production of wide variety of proteins. In a scale-up study, it was shown that *S. lividans* is convenient for recombinant production of *Mycobacterium tuberculosis* antigen, APA [13]. Elucidating the complex metabolic mechanisms affecting protein synthesis in *S. lividans* improved the recombinant protein production in this industrially important species [14]. Efficient extracellular secretion mechanism of *S. lividans* renders this bacterium as a prominent synthesis machinery [15] for the production of wide range of

heterologous proteins [16] and omics-based researches have been conducted to improve the productivity of this organism [17].

2.2. Bacillus *spp.*

The other production hosts for recombinant proteins are *Bacillus* species because of their attractive secretion system exporting the proteins into the extracellular environment [18]. This secretion system has been utilized in many studies for recombinant protein production in different *Bacillus* species such as *B. subtilis* [19–21], *B. brevis* [22,23] and *B. megaterium* [21,24]. *Bacillus* species are good choice to produce proteins especially for industrial or pharmaceutical purposes. Some appropriates such as capability of extracellular protein production, availability to high-cell density fermentation, and easy adaptivity to genetic modifications render different species of *Bacillus* as efficient expression systems for industrial enzyme production [25,26]. Many of *Bacillus* species are known as the main sources of proteases which can be used in different fields such as food, beverage, leather, textile, detergent or pharmaceutical industries [27]. Innovative strategies are carried out to develop different *Bacillus* expression systems which produce high yield of recombinant proteins with expanded application fields. The attempts for optimization of secretion pathways, enhancement of RNA and protein stability, facilitating cell growth conditions, to streamlining bacterial genome and engineering transcription regulation mechanism were reported [28].

2.3. Lactococcus lactis

Lactococcus lactis is known as a convenient choice for fermentation, and generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) [29]. *L. lactis* serves as an attractive platform for recombinant protein production both for food-grade [30] and pharmaceutical [31] proteins. Besides use as a recombinant production platform for vaccine antigens [32], there are interesting efforts on using the recombinant form of *L. lactis* as oral or mucosal vaccine delivery system [33–37].

2.4. Escherichia coli

Strains of *Escherichia coli* are used most widely for recombinant protein production over years. Some properties of *E. coli* such as its (i) well characterised genetic structure, (ii) suitabilitiy for genome engineering to gain different functions, (iii) cost-effectiveness, (iv) adaptivity to high level protein production protocols make this bacterium as one of the favorite synthesis machinery for the production of recombinant proteins that do not need complex post-translational modifications for functional activity [38]. *E. coli* is the most popular among other bacterial host for the production of pharmaceutical proteins and it was the host for somatostatin as the first recombinant protein [39]. As a recombinant protein production host, *E. coli* includes a great number of recombinant strains having different pros and cons relative to each other [40]. Therefore, it is crucial to choose the right *E. coli* strain for high yield protein production, as well as optimization of other incubation conditions. There are numerous protein products produced in different *E.coli* strains for medical and non-medical applications [41–44].

2.5. Pseudomonas *spp.*

Due to the rapid growth rate and appropriate secretion system, *Pseudomonas* species, especially *P. fluorescens*, might be shown as alternative expression hosts among the other recombinant protein production systems. Jin et al. [45] showed the capability of *P. fluorescens* for recombinant production of human granulocyte colony stimulating factor, a cytokine controlling the production, differentiation and function of granulocytes, in its active and soluble form. In addition, identified signal sequences of *Pseudomonas* species that facilitate the transport of expressed recombinant proteins to the periplasmic space are of interest to improve protein stability, solubility, folding and purification processes [46]. Besides, *P. aeruginosa* and *P. putida* serve as potential and efficient platforms for the recombinant production of several proteins for industrial and medical applications, as well [47].

2.6. Corynebacterium glutamicum

Corynebacterium glutamicum is another bacterial host for recombinant protein production. This bacterium has been generally used for production of amino acids like L-glutamic acid and L-lysine, for long years [48,49]. In addition to adaptation capacity to culture medium and low protease activity, its effective secretion pathways make this species as an alternative choice for heterologous protein production [50]. Moreover, *C. glutamicum* was shown as a potential production host for pharmaceutical proteins at large-scale [51]. There are various studies reporting the importance of *C. glutamicum* for production of enzymes, antibody fragments, or saccharides [52]. Also, other studies have reported on the development of this bacterium or its growth conditions for industrial uses [53,54].

3. Promoters and gene expression

A promoter is an element responsible for initiation and regulation of transcription of the gene cloned into an expression plasmid [55], and also coordination of biosynthetic pathways controlled by multi-genes [56]. In a plasmid vector, this DNA region is located on the upstream of gene encoding the protein of interest. A promoter contains nucleotide sequences recognized by transcription factors and RNA polymerase (Figure 1). Concerning the expression of recombinant genes cloned on its downstream region, it is crucial to choose a compatible promoter with endogenous RNA polymerase of host cell system. Therefore, suitable promoters should be selected for prokaryotic or eukaryotic host cell systems.

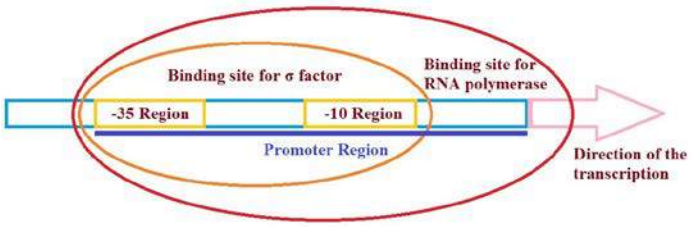

Figure 1. Schematic representation of the conserved -35 and -10 boxes as well as the binding sites for σ factor and RNA polymerase in a prokaryotic promoter region.

Promoters can be at the “on” formation without existence of a stimulant for activation. However, regulated promoters need a specific tissue, time, physical or chemical stimulants to switch from “on” to “off” formations or vice versa (Figure 2). Although the complex control mechanism of the cell on promoters, unwanted transcription, regarded as “leaky”, occurs in some situations. This phenomenon may lead to loss of viability of cells, production yield and stability of protein to be produced, when cloned gene or its products are toxic to expression host. To overcome leaky transcription, many of synthetic promoters regulated tightly were obtained [55,57]. These types of prokaryotic promoters are chosen to increase stability and production yield of proteins for recombinant production. Strong promoters are generally used in recombinant protein production owing to the vital role of promoter strength on productivity. In general, strong promoters require an inducer for binding of RNA polymerase for transcription initiation. Therefore, it is desirable to achieve an efficient expression system harboring a strong promoter, non-leaky expression and requires cheap and non-lethal inducers for overproduction of recombinant proteins. For these reasons different studies for development of novel inducers [58,59] or auto-inducible promoters [59,60] which are useful for industrial protein production hosts have been reported. Some of the promoters for prokaryotic expression systems are listed in Table 2.

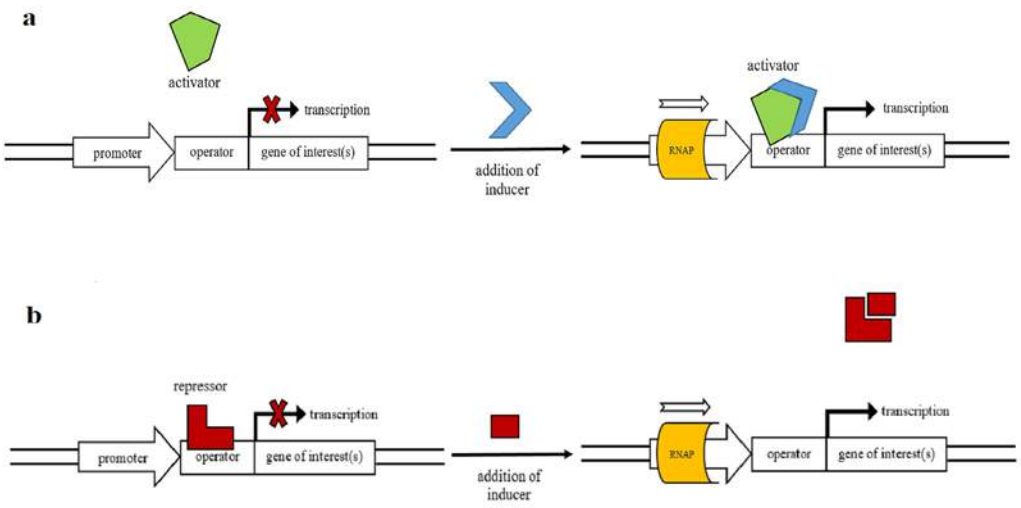

Figure 2. Positive and negative induction mechanisms for regulation of inducible promoters. In positive induction mechanism, (a) the promoter is inactive due to the absence of activator-promoter binding (OFF state). Upon the stimulation by inducer, the activator binds to specific DNA sites and initiates transcription (ON state). In negative induction mechanism, (b) a repressor is bound to specific DNA site and transcription is blocked (OFF state). After the stimulation via an inducer, repressor is released and the transcription is initiated (ON state).

A promoter sequence behaves as a functional program including some of major steps: (i) recognition by RNA polymerase; (ii) isomerization of the initial complex; (iii) transcription initiation; (iv) transition to elongation complex and clearance of the promoter [61]. Commonly used promoters can be classified in three different groups (i) constitutive promoters, (ii) inducible promoters, and (iii) stationary phase promoters. The constitutive promoters may be described as promoters that are active

under *in vivo* conditions [62]. Transcription of the gene cloned under some types of constitutive promoters, such as T3, T7 and SP6 promoters, requires RNA polymerase enzymes recognizing specific sequences on their cognate promoters for transcription [63]. Inducible promoters may be divided into two subclasses known as inducer-dependent promoters and auto-inducible promoters, based on their requirement to inducer for subsequent gene expression. The *spac* and *xyl* promoters are examples of inducible promoters induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) and xylose, respectively [64]. Although inducible promoters show effective overexpression of the gene of interest, they are not widely accepted in practice for industrial protein production because of the cost of the inducer and associated host-toxic properties. Whereas expression vectors with auto-inducible promoters, that do not require an inducer in culture medium for transcription initiation, may be referred as suitable for industrial-scale protein production [65]. Some of the proteins synthesized in the stationary phase of bacterial growth are vital due to contribution to cell viability. The promoters responsible for expression of these proteins are called stationary-phase promoters. These promoters show their activity at the stage of stationary phase and less or no activity in growth phase, namely exponential (log) phase [66], and they are suitable for large-scale protein production due to both the cost-effectiveness from no requirement of an expensive inducer and absence of the possible toxic effects of inducers. Hence, attempts on identification and characterization of stationary-phase promoters are effective for enrichment of gene expression under these promoters [67,68]. Comparative studies on promoters reveal the influence of promoter strength and activity on protein production [69,70]. Since the strength of a promoter can be determined as measure of transcription initiation frequency, the affinity of RNA polymerase to promoter sequence may be accepted as the main effective parameter [71]. Based on this frequency, promoters are considered as weak or strong. A weak promoter generally gives low transcription, but a strong promoter produces more. However, strong promoters are not always good choices when the level of basal transcription of the gene encoding a protein of interest is deleterious or toxic for host cells [72]. Besides total amount of produced recombinant protein, the amount of protein produced in soluble form is also important. For this reason a weak promoter can enhance the solubility, while a strong promoter can enhance the amount of total protein [73].

Table 2. Examples of promoters used in prokaryotic organisms.

Promoter	Strain	Intended use	Reference
T7	*E. coli* Rosetta-gami B (DE3)	Recombinant production of potassium channel blocker protein	[74]
	E. coli BL21 (DE3) CodonPlus	Recombinant production of *Pseudomonas aeruginosa* exotoxin A	[75]
T7*lac*	*E. coli* BLR (DE3)	Expression of recombinant asparaginase fused to *pelB* leader sequence	[76]
	E. coli BL21(DE3) pLysS and Rosetta 2(DE3) pLysS	Engineering of *E. coli* producing 1,3-propanediol from glycerol	[77]
rpoS	*E. coli* DH5α	Engineering of *E. coli* strain to produce 1,3-propanediol directly from glucose	[78]

Continued on next page

Promoter	Strain	Intended use	Reference
PrhaBAD	*E. coli*	To improve the production of membrane and secretory proteins in *E. coli*	[79]
mmsA	*Pseudomonas denitrificans*, *E. coli* and *P. putida*	To develop a biosensor for 3-hydroxypropionic acid	[80]
trc	*Corynebacterium glutamicum*	To evaluate the effect of polyhydroxybutyrate synthesis genes on L-glutamate production in *Corynebacterium glutamicum*	[81]
tetA	*E. coli* K-12 and B strains	Production of murine immunoglobulin Fab fragments	[82]
tac	*Corynebacterium glutamicum*	Heterologous production of amylase	[83]
lac	*Escherichia coli* RB791	Optimizing the expression of a monoclonal antibody fragment	[84]
tacM	*Corynebacterium glutamicum*	Sec pathway-dependent production of α-amylase	[85]
P_L	*E. coli* BL21	Investigating metabolic and transcriptional responses to temperature during synthesis of pre-proinsulin	[86]
araBAD	*E. coli* DH10B	Production of TrfA replication protein	[87]
cspA	*E. coli* BL21	To elucidate the functional alteration of the recombinant hybrid chitinases composed of bacterial and insect's domains	[88]
cspB	*Corynebacterium glutamicum* YDK010	Production of human epidermal growth factor	[51]
phyL	*Bacillus licheniformis*	α-amylase and xylanase production as reporter proteins in development of an auto-inducible promoter	[59]
NBP3510	*Bacillus subtilis*	Production of intracellular (BgaB, sfGFP) and extracellular (MPH, Chd) proteins	[68]
P43	*Bacillus subtillis* WB800N	Trehalose synthetase expression for trehalose production	[89]
P*spac*	*Bacillus subtillis*	β-galactosidase production	[90]
P_{170}	*Lactococcus lactis*	Production of nuclease from *Staphylococcus aureus*	[91]
P*grac*	*Bacillus subtilis*	Recombinant human bone morphogenetic protein 2 (rhBMP2)	[92]
trp	*E.coli* K-12	Expression of different foreign genes	[93]

4. Engineering of the promoters for production of recombinant proteins

Many innovative approaches stand out for discovery or modification of prokaryotic [94] and eukaryotic [95] promoters. Obviously the identification of bacterial promoters is important to achieve higher yields of recombinant proteins produced in bacterial cell factories. Promoter engineering is a strategy to create large number of promoter libraries and to regulate promoters with different strength and functions. Many studies based on mathematical prediction, randomization of

promoter sequences and hybridization of promoters are used for promoter modifications [56]. Although this strategy is one of the main topics of metabolic engineering and synthetic biology, it is also practical for overexpression of recombinant proteins. In this regard, bioinformatics serves as a useful tool for discovery of bacterial promoters [96]. Researches on identification and development of novel promoters mainly consist of three strategy: (i) discovery of novel promoters by microbial genome screening [97,98], (ii) constructing of broad libraries of artificial promoters [94,99] and (iii) editing the core region of existing promoters [100].

Sequencing the genomes of different bacteria is an applicable way leading to discovery of novel promoters [101]. For this purpose, transcriptome mining may be a convenient choice as a bioinformatics tool based on genome screening, for identification of novel and stronger promoter(s) from the transcriptome data of the strain of interest. By this approach, Meng et al. [102] reported *B. subtilis* as an appropriate expression host for the production of pullulanase, a starch hydrolysis enzyme, at industrial quantity. In their study, four strong promoters were identified from *B. subtilis* transcriptome. New recombinant strains with individual promoters or combinations thereof were generated. Results from the studies on screening of endogenous promoters lead to efficient engineering of strains. Newly identified promoters can effectively optimize the molecular pathways and improve the productivity in strain of interest as well as increasing the industrial protein production [103]. High-throughput promoter screening and identification studies may be useful for developing novel expression system for research and biotechnological applications [104].

Development of synthetic promoters has also importance for engineering of strains to produce large-scale protein production. In a study for engineering a fully synthetic promoter for constitutive protein expression, Yim et al. [105] constructed synthetic promoter libraries via green fluorescent protein as a reporter and then isolated the potential ones by fluorescence-activated cell sorting (FACS)-based high-throughput screening strategy. Different proteins were produced in large-scale to verify activity of synthetic promoters. In line with the production yields, usefulness of P_{H36}, the strongest synthetic promoter in the study, in large-scale fed-batch production was proved. Results obtained from this study are important in the aspect of showing the effect of promoters and strength thereof for engineering a strain to extend its production capability and productivity. Zhou et al. [68] achieved overproduction of intracellular (BgaB, sfGFP) and extracellular (MPH, Chd) proteins from a single copy expression cassette in *B. subtilis* via modification of the core regions and upstream sequences of existing stationary phase-dependent promoter P_{ylb} to obtain high yield transcription. Production experiments showed that the resulting promoter, NBP3510, was suitable both for overproduction of intracellular and extracellular proteins. Besides, studies relies on high-throughput screening of mutant promoters are available for optimization of promoter strength to produce the proteins in active form and with increased levels [106]. Moreover, it is possible to construct broad-spectrum promoters functional in diverse microorganisms. Since identical housekeeping sigma factors σ^{43} and σ^{70} recognize conserved -35 and -10 boxes, Yang et al. [107] designed a broad-spectrum promoter, P_{bs}, for *E. coli*, *B. subtilis* and *Saccharomyces cerevisiae* using a strong synthetic promoter platform (P_{min}) for *S. cerevisiae*. They also used random mutagenesis to obtain three different variants, P_{bs1}, P_{bs2} and P_{bs3}, differing in strength.

Due to the vital role of promoters in gene expression, many attempts focus on discovery, development or improving the strength of promoters. In addition to other promoter-based researches, Yang et al. [108] developed phosphorothioate-based ligase-independent gene cloning method, named as PLICable-pET promoter toolbox, for evaluation of ten IPTG inducible promoters (T7, A3, *lpp*, *tac*,

pac, Sp6, *lac*, *npr*, *trc* and *syn*) to determine the most suitable promoter for high yield production. By the application of PLICable-pET tool box, an increment on the production levels of different proteins was reported. They suggested this system to screen suitable promoter in a single cloning step quickly and effectively.

In another approach, a plasmid, called as promoter trap vector, including a multiple-cloning site at the 5' end of a marker gene without a promoter sequence can be used in promoter identification studies. In this system, it involves cloning of a couple of unknown DNA fragments and a reporter gene, and subsequent monitoring for reporter gene expression reveal novel promoters [109]. Yang et al [110] developed a promoter trap system which can shuffle and recombine different DNA fragments and screen strong promoters at the same time. In their study, partially digested genome of *B. licheniformis* was ligated to the promoter trap vector and then transformed into *E. coli* DH5α. Subsequently, recombinant strains including promoter fragments were screened. Approximately 1000 colonies potentially carrying the desired promoter were obtained using this vector system. Strength of promoters was compared based on productivity of β-galactosidase both in *E. coli* and *B. subtilis*. This study demonstrated the usefulness of promoter trap vectors for developing an artificial double promoter system consists of P_{luxS} and a hybrid promoter from *B. licheniformis*. They suggested simultaneous shuffling and recombination of DNA fragments and adoption of a high throughput screening as the advantage of the trap system to create new promoters.

One-to-one promoter comparison studies identifying suitable promoters to increase the productivity of the system may guide to generate an efficient protein production platforms [108]. In addition, this comparison studies may be helpful to show different effects of common promoters on soluble and total protein yields, in host-dependent or independent manner [73]. There are many components which should be set to obtain optimized production platform for recombinant proteins, such as modification of genetic construct [111], determination of the best host-vector combination [76], or altering the growth parameters like concentration of specific medium ingredients [112]. The combination of these parameters may be efficacious to achieve high yields of protein production. Among the choices aforementioned above, Yim et al. [111] showed the effect of genetic circuit modification to obtain desired protein levels. They examined the effects of signal sequences, codon-optimization, promoters, untranslated regions and transcriptional terminator on the yield of protein secreted into medium and significant increase was reported for secreted protein. As a result of this study, a fully synthetic promoter, H36, stands out for obtaining the highest level of protein. This promoter enhanced the production quantity 10 to 20 folds in comparison to other promoters, pSodM2 and pTrcM3, respectively. This study revealed the contribution of right promoter selection to development of efficient expression systems. A number of studies revealed the importance of correct combinations of production host, promoter and signal sequences for improvement of protein production [113]. Promoter engineering approaches are also employed for the recombinant protein production in unusual hosts. Shen et al. [114] obtained a suitable promoter for the production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] in *Halomonas bluephagenesis*. They used saturation mutagenesis approach to obtain a library for the promoter core region of porin gene (P_{porin}). The best promoter was used for the expression of *orfZ* gene in *H. bluephagenesis* and enhanced production of P(3HB-co-4HB) was obtained. Additionally, combinations of promoters and signal sequences might be utilized for on-line analysis of bacterial host responses during recombinant protein production [115]. Taken together, these results highlight the impact of promoter strength and combination on heterologous protein production efficiency.

5. Production of recombinant proteins using double promoter systems

Some researchers prefer co-expression strategies to produce multi-protein complexes by more effective manner [116]. There are two commonly used method for co-expression of proteins: dual vector systems and bicistronic vector systems [117]. In dual vector system, two different genes encoding the proteins of interest are cloned into two separate expression plasmids, and these two plasmids are introduced into a single host cell. However, the problem of domination of one plasmid to the other in terms of copy number may occur when dual vector systems are used [118]. In bicistronic vector system, different genes are cloned under a single promoter. In theory, expression levels of these genes are comparable since both of them have their own ribosome binding unit but practically, the gene away from the promoter sequence is expressed less [119]. In this case, the transcription level of the second gene located away from promoter can be increased by adding a second promoter in the upstream region of this gene. Thus, the production of protein encoded by second gene can be enhanced [119,120]. Double promoter plasmid systems have been used over years [121]. Kim et al. [120] compared the productivity of traditional bicistronic vectors and double promoter vectors, and demonstrated that the double promoter system enhanced the protein production four to nine folds. They also suggested that combination of more promoters may enhance the amount of produced protein.

In Figure 3, a traditional bicistronic vector system and a double promoter vector system were illustrated schematically. Öztürk et al. [122] classified double promoter systems in two major groups, as consecutively (CNT)- and simultaneously (SMT)-operating ones, based on their mechanism of operation. According to this classification, SMT-operating double promoter systems can also be subdivided into three different groups as carrying two same promoters, a promoter and its variant, and two different promoters.

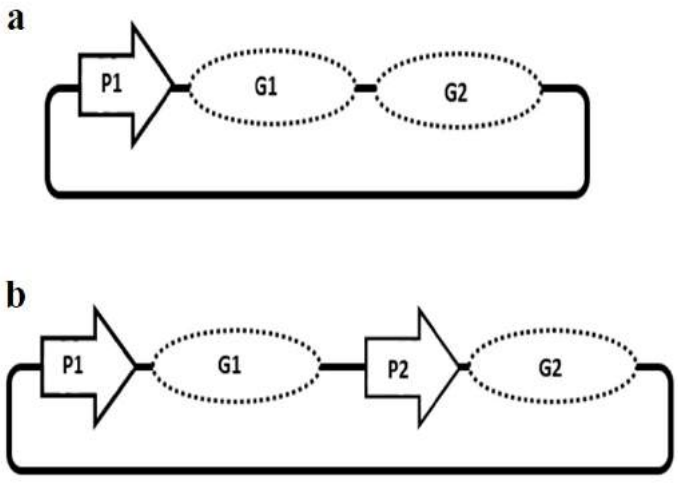

Figure 3. Schematic illustration of a traditional bicistronic vector system (a) and a double promoter vector system (b). In double promoter system P1 and P2 can be consecutively or simultaneously operating, identical or non-identical promoters. P1: promoter 1; G1: gene 1; P2: promoter 2; G2: gene 2.

Ray et al. [123] used a dual promoter expression plasmid including two different inducible promoters and secretion genes in *E.coli* to take advantage of extracellular expression of the protein of interest such as achieving enhanced stability, proper folding, higher production and purification yields. Co-expression of secretion factors in dual promoter-expression plasmid maximized the extracellular production of salmon calcitonin protein. Similarly, Tao et al. [124] constructed a dual-promoter expression plasmid for recombinant production of phospholipase D (PLD) from *S. halstedii* in *S. lividans*, consisting of inducible (Ptip)/constitutive (PermE*) promoters. For this, a strong constitutive promoter, the erythromycin resistance promoter PermE*, was inserted downstream of the inducible Ptip promoter, and they could reduce both the dependence on expensive inducers and production costs.

Liu et al. [125] combined the suitable signal peptide detection and promoter strength comparison studies to establish practical and optimal expression system for recombinant production and secretion of alkaline serine protease (BcaPRO) from *B. clausii* in *B. subtilis* WB600. Plasmids carrying signal peptide and BcaPRO gene were combined with a single promoter, as well as dual or triple promoters. Engineered expression system involving two promoters (P_{Bsamy}-P_{Baamy}), the signal peptide (SP_{Dac}), and BcaPRO gene was found effective for high-level production of the protein of interest. In parallel, Guan et al. [126] evaluated the optimization of promoters and signal peptide combinations for secretory production of aminopeptidase (AP) in *B. subtillis*. After screening the library composed of approximately 20 Sec-type signal peptides, YncM was determined as the most effective one for enhancing the secretion. Later, AP and YncM were cloned to a series of expression plasmids harboring single or dual promoters. Fermentation process showed that secretory production of AP was maximized by expression vector containing YncM and AP under the P_{gsiB}-P_{HpaII} promoters. This study suggested that combination strategy of double promoter systems and secretory signal peptides can lead to advantageous results for recombinant secretory protein production. Moreover, Zhang et al. [127] developed a novel dual promoter expression vector for production of industrial relevant enzymes in *B.subtilis*. To obtain a dual promoter plasmid, a screening step was conducted with single-promoter plasmids encoding β-cyclodextrin glycosyltransferase (β-CGTase). In this step, plasmid with P_{amyQ} promoter showed the highest activity. Next, dual-promoter vectors were constructed based on P_{amQ} promoter and evaluated same as single-promoter vector screening study. Production studies showed that the novel constructed dual promoter plasmid, P_{HpaII}-P_{amyQ}, can produced β-CGTase and other industrial enzymes such as pullulanase effectively both in shake flasks and 3 L fermenters. Therefore, this dual promoter system was suggested as a potential applicable system for industrial purposes. In another study based on production of industrial enzyme, pullulanase in *B. subtilis*, Liu et al. [128] developed a mutant *Bacillus* sp. by deletion of multiple proteases. After screening and comparison studies of single- and multiple-promoters, the dual promoter system, P_{amyL}–P_{spovG}, was found supportive for increased extracellular protein production. Combination of host strain construction and promoter optimization, they successfully produced the enzyme pullulanase in active form and high yield. Their works demonstrated the potential applications of double-promoter plasmid systems for industrial protein production.

Success and importance of dual promoter-based vector systems on rapid, economical and high titers production of antibodies or fragments of these complex biomolecules have shown in different types of host cells [120,129]. Lueking et al. [130] constructed a system for inducible production of recombinant proteins both in *Pichia pastoris* and *E. coli*. In their dual promoter system they combined the eukaryotic and prokaryotic promoter elements so that *E. coli* T7 promoter region with

the ribosome binding site was located on downstream of alcohol oxidase promoter (AOX), a strong yeast promoter. According to comparison study for human protein expression in eukaryotic and prokaryotic hosts, it was obvious that dual promoter expression system reduces workload and ensures an economic platform for production and purification of recombinant proteins both without subcloning steps. Similarly, Sinah et al. [131] suggested the dual promoter vectors as useful tools for cloning, expression, and purification of proteins both in eukaryotic and prokaryotic host or for studying post-translation modifications.

6. Conclusion

Recombinant proteins are very valuable products for a wide range of usage areas. Due to their economical importance, conditions such as cost-effective and high-yield production of recombinant proteins in diverse hosts require optimization of many factors including gene expression. Promoters are among the key factors regulating gene expression. Therefore, utilization of an efficient promoter system is vital for recombinant protein production. The findings of many studies showed that engineering of promoters to obtain an optimal expression system as well as utilization of dual promoter-based vector systems are among the efficient strategies for enhanced production of recombinant proteins.

Conflict of interest

The authors declare no conflict of interest.

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