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Chapter

Safety Aspect of Recombinant Protein Produced by *Escherichia coli*: Toxin Evaluation with Strain and Genomic Approach

Iman Permana Maksum, Ahmad Nabiel, Safri Ishmayana and Ukun M.S. Soedjanaatmadja

Abstract

Escherichia coli is a Gram-negative bacteria which is well known for its pathogenic properties that can cause serious food poisoning, mostly indicated by diarrhea or other severe symptoms. Despite of its well-known properties due to its ability to produce toxin, most of *E. coli* strains are harmless and even beneficial especially in recombinant protein production. This bacterium is suitable for protein recombinant host since it has rapid growth, high expression rate, and well-known genome. Various proteins have been produced using *E. coli* expression systems, with therapeutic protein for medical application being the most notably produced. Apart from that, our group succeeded in producing beta galactosidase from a wild type *E. coli* strain B130. Furthermore, recombinant human serum albumin was successfully produced using *E. coli* strains BL21 (DE3). However, studies on *E. coli* toxin contamination in recombinant protein productions, strains, and genomic comprehension are indispensable, particularly in therapeutic protein. Therefore, this chapter will discuss the safety aspects of recombinant therapeutic proteins in terms of toxin contamination by strain and genomic approaches.

Keywords: *E. coli*, genomic maps, recombinant therapeutic proteins, toxin

1. Introduction

Escherichia coli is a member of *Enterobacteria* family which can be found in gastrointestinal tracts [1–3]. In general, it is well known to cause broad diseases, including gastrointestinal problems. Aside the fact that *E. coli* was normal to be found in colon, a number of its strains were discovered with the ability to produce toxins. Shiga toxin *E. coli* (STEC) and enterotoxin *E. coli* (ETEC) were groups of *E. coli* strains that have the ability to produce toxin that may cause several diseases, such as diarrhea [1, 4, 5].

Although *E. coli* may cause numerous gastrointestinal diseases; in fact, strains that are responsible for pathogenic properties were relatively minor in numbers. Furthermore, *E. coli* was considered harmless and even useful as a host for producing recombinant proteins. Even this bacteria becomes favorite host chosen in industrial and medical applications since it has rapid growth, well-characterized

gene, and its ability to grow under aerobic and anaerobic system, and facilitates to form high cell density culture (HCDC) [6–8].

The discussion about advantage in producing recombinant proteins and worries of toxins of *E. coli* is like talking about two opposite sides of a coin. This will certainly raise a question "Is it safe to produce recombinant protein in *E. coli*? Will it be toxin-free contamination?" Therefore, this chapter will discuss the safety aspects of recombinant protein produced by *E. coli* against toxins using genomic and strains approach.

2. Toxin produced by *Escherichia coli*

Several pathogenic *E. coli* strains are known to be responsible for broad diseases, from mild to complicated cases. It is varying from mild diarrhea, hemorrhagic colitis, to hemolytic uremic syndrome. Among the pathogenic strains, STEC is an example of common strains which occupy high number in *E. coli* serotypes that produce toxin called Shiga toxins (Stx) [1, 2, 5, 9–11]. While STEC is a common pathogenic example, it belongs to a larger group named enterohemorrhagic *E. coli* (EHEC); also, there still exist numerous pathogenic *E. coli* and cause different diseases and complications. Pathogenic *E. coli* were classified in **Table 1** along with its diseases they caused and virulence factors [1].

Considering the number of pathogenic *E. coli*, it is useful to classify the toxins' properties and structure. It will be convenience to determine whether the toxins belong to organic compound or peptide-based structure; therefore, we could analyze contamination probabilities in terms of producing recombinant protein. Most of the virulence factors stated in **Table 1** were protein attached in bacterial membrane with the role of adhesion or recognition to host cell [12]. Meanwhile, shiga toxin, heat-stable and heat-labile toxin, and other cytotoxins were protein released by pathogenic *E. coli*. These toxins have specific receptors to induce invagination to the host cell, while their virulence mechanism also differs depending on the nature of each toxin and their molecular target [4].

STEC serotypes vary and differ in number of incidences, although the O157:H7 is a serotype considered to be responsible of numerous outbreaks. Shiga toxin occupies AB5 structure (see **Figure 1**), the catalytic subunit A (StxA) and homopentamer of subunit B (Stx B) as recognition site to globotetraosylceramide (Gb3/Gb4), which are present in the host cell surface, which leads to invagination of the toxin. STEC can produce either Stx1 (Stx1 and Stx1c), Stx2 variant (Stx2, Stx2c, Stx2d, Stx2e, and Stx2f) or range combination of both variants [4, 12]. Once invagination succeeds, catalytic subunit A would disrupt cell metabolism by inhibiting elongation factor-dependent aminoacyl tRNA binding (see detailed mechanism in [4]). The highly specific RNA N-glycosidase activity cleaves adenine base in eukaryotic ribosomal RNA, precisely at 28S subunit on the α-sarcin loop located in position 4324 [4].

Meanwhile, heat-labile (LT) and heat-stable (ST) toxin belong to ETEC groups. Nevertheless, LT enterotoxin shares similar structures to Stx which occupy AB5 conformation. Subunit A acts as a toxin by binding to its receptor, guanylyl cyclase C (GC-C). The interaction will activate guanine nucleotide protein Gsα by ADP-ribosylation, which trigger stimulation of secretion by cAMP-dependent mechanism. Elevated numbers of cAMP cause CTFR channel to secrete water and ions, thus generating diarrhea [3]. By contrast, ST structure is relatively simple. The STa class was made up with 18–19 cysteine-rich amino acids, while STb has 48 amino acids. ST virulence acts by triggering secretion of water and ions by triggering signaling cascade through guanylyl cyclase C (GC-C) in intestine [13, 14]. The structure of both ST and LT is shown in **Figure 2**.

| Pathogenic	Diseases	Virulence factors	Reference
Enteric *E. coli*			
Enteropathogenic *E. coli* (EPEC)	Diarrhea in children	Bfp, intimin, LEE	
Enterohaemorrhagic *E. coli* (EHEC)	Hemorrhagic colitis, hemolytic uremic syndrome	Shiga toxins, intimin, Bfp	
Enterotoxigenic *E. coli* (ETEC)	Traveler's diarrhea	Heat-labile and heat-stable toxins, CFA	
Enteroaggregative *E. coli* (EAEC)	Diarrhea in children	AAFs, cytotoxins	
Enteroinvasive *E. coli* (EIEC)	Shigellosis-like	Shiga toxin, hemolysin, cellular invasion, Ipa	[1]
Diffusely adherent *E. coli* (DAEC)	Acute diarrhea in children	Daa, AIDA	
Adherent invasive *E. coli* (AIEC)	Crohn's disease-associated	Type 1 fimbriae, cellular invasion	
Extraintestinal *E. coli*			
Uropathogenic *E. coli* (UPEC)	Lower urinary tract infection and systemic infection	Type 1 and P fimbriae, AAFs, hemolysin	
Neonatal meningitis *E. coli* (NMEC)	Neonatal meningitis	S fimbriae, K1 capsule	
Avian pathogenic *E. coli* (APEC)	Food-borne source disease	Type 1 and P fimbriae, K1 capsule	

AAF, aggregative adhere fimbria; AIDA, adhesion involved in diffuse adherence; Bfp, bundle-forming pili; CFA, colonization factor antigen; Daa, diffuse adhesin; Ipa, invasion plasmid antigen; LEE, locus for enterocyte effacement.

Table 1.
Classification of pathogenic E. coli*.*
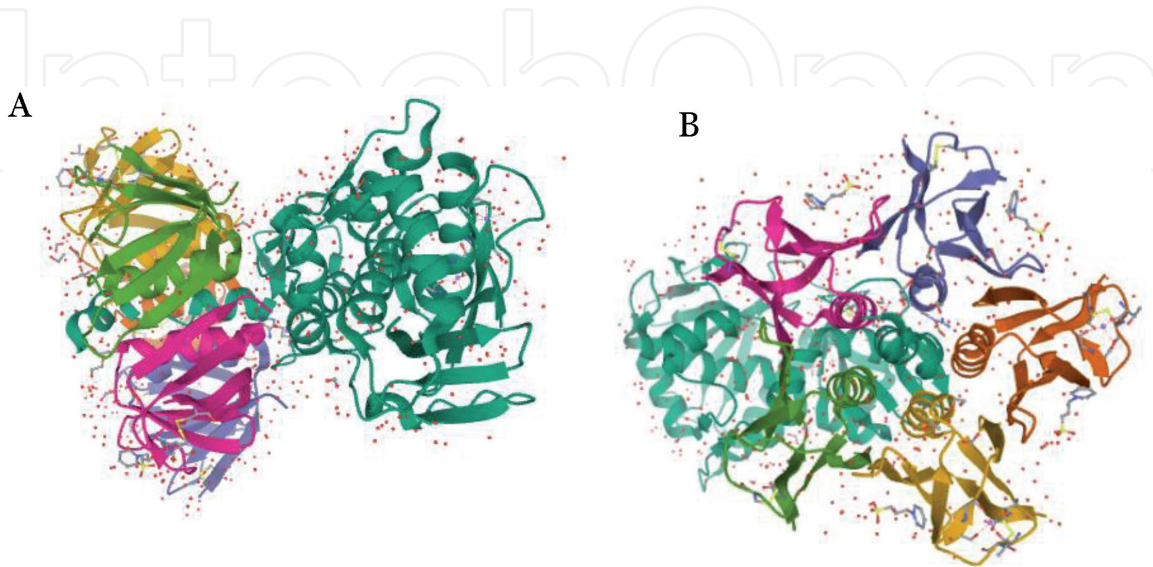
Figure 1.
Crystal structure of shiga-like toxin in E. coli *(PDB: 1R4P). (A) Transversal view of Stx subunits, StxA represented as teal-colored chain, while StxB presented in rainbow color; (B) axial view of homopentamer StxB [3].*
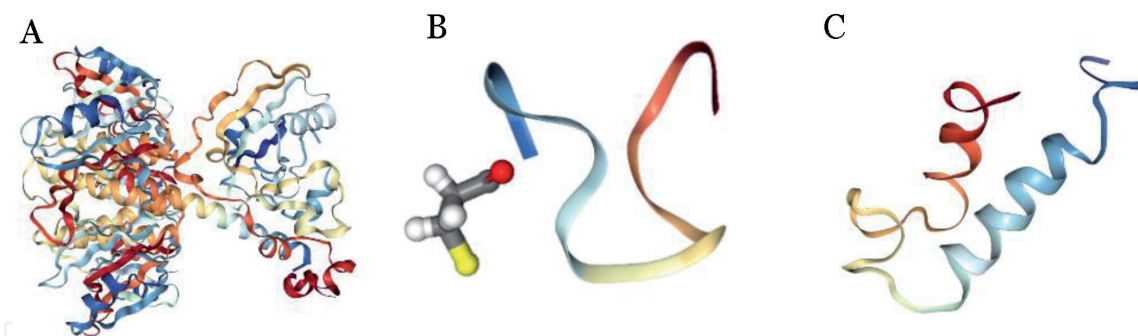
Figure 2.
Enterotoxin secreted by ETEC. (A) Heat-labile enterotoxin (PDB: 1tii) [16]; (B) heat-stable enterotoxin, STa class (1etn) [17]; (C) heat-stable enterotoxin, STb class (1ehs) [18].

The fact both STEC and ETEC toxins (Stx and LT, and ST, respectively) are peptide based elucidates its origin that were genetically listed in their DNA. These toxins were made under central dogma of protein synthesis. Therefore, analysis through genomic approach on recombinant *E. coli* host is possible to be conducted.

3. *E. coli* as host for recombinant protein expression

The production of recombinant proteins in microbial systems was started in 1970 and continued to boom in 1980 with the production of insulin. There is no doubt that this method has revolutionized and widened the field of biochemistry [19]. The ability to express large quantity of protein with less effort, relative to manual synthesis, allows industrial processes to produce in commercial scale. However, several considerations should be discussed before executing the production such as, appropriate vector, location of the protein of interest (whether as soluble fraction or inclusion bodies), optimum condition (pH, medium, temperature, aerobic/anaerobic system), genetic design for convenience of purification, and at the top of it, microbial selection [7, 8, 20].

E. coli become preferred microbes in terms of recombinant protein host among researchers and industrial use. The simplicity of its expression system, compared to other higher level organism, and large quantity of well-characterized genomic database offer advantages in constructing the vector to be used [20]. A plenty number of research regarding *E. coli* also become an advantage to give amount of consideration of various expression conditions. Nevertheless, *E. coli* expression system has limited post-translational modification, which means that some proteins that require modification, such as alkylation or glycosylation, may not be perfectly expressed in *E. coli*. However, several strains of *E. coli* have the ability to perform specific post-translational modification [19, 21]. Therefore, we provide a simple summary on recombinant proteins produced by *E. coli* along with strains and expression strategies in **Table 2**.

Among recombinant proteins mentioned in **Table 2**, hEGF and hPT-2 are examples of therapeutic protein. Regarding its use in medical interests, therapeutic proteins produced in *E. coli* have to be safe for administration into human bodies; therefore, purification steps and any contaminants present become a huge concern in producing recombinant protein. Idetifying location of protein target is a prominent fundamental to determine source of contamination and to predict any possible contamination. Understanding the protein location also helps with the purification strategies needed to separate contaminants, specifically toxins, with the result that highly pure proteins were recovered. Choi et al. [29] through **Figure 3** classify locations of protein expressed in *E. coli* and its general purification steps needed.

Recombinant proteins	Strain	Expression strategies	Ref.
α-Cyclodextrin glycosyltransferase	BL21 (DE3)	Extracellular, using OmpA constructed on pET-20b(+) vector	[22]
P64k	*E. coli* K12 GC366	Soluble intracellular, using pM-152 vector	[23]
SpA-βgal (*Staphylococcus* protein A-*E. coli β*-galactosidase)	*E. coli* RR1 LacZ	Soluble intracellular as fusion protein	[24]
Human prethrombin-2 (hPT-2)	*E. coli* TOP10F'	Fusion protein with intein, CBD-*Ssp* DnaB-hPT-2	[25]
Human prethrombine-2 (hPT-2)	*E. coli* BL21 (DE3) ArcticExpress	Soluble intracellular using pTWIN1	[26]
Human epidermal growth factor (hEGF)	*E. coli* BL21 (DE3)	Extracellular using TorA signal peptide constructed on pD881 vector	[6]
Human epidermal growth factor (hEGF)	*E. coli* BL21 (DE3)	Extracellular, using OmpA constructed in pD881 vector	[27]
Human serum albumin	*E. coli* BL21 (DE3)	Extracellular, using TorA constructed on pD881 vector	[28]
Pyrococcus furiosus α-amylase (PFA)	*E. coli* DH5α	Soluble protein, designed with co-expression of prefoldin, chaperonin HSP60, and sHSP	[29]
Hepcidin	*E. coli* BL21 (DE3)	Inclusion bodies, fused with His-tag	[30]
Keratinase	*E. coli* AD494(DE3) pLys	Intracellular expression using His-tag	[31]
Pig liver esterase (γ-PLE)	*E. coli* origami (DE3)	Soluble intracellular, using Strep-tag and combination of His- and Trx-tag	[32]

Table 2.
Summary of recombinant protein using E. coli *expression system and its expression strategy.*

The distinction of protein location is affected by either the nature of the expression system or the protein construction design. Both extracellular and intracellular strategies on expressing protein give its own advantages and disadvantages. Extracellular expression offers simple purification, improved folding, and soluble products. This strategy can be achieved using signal peptide, co-expression with phospholipase, or co-expression with chaperon [33, 34]. In contrast, intracellular expression prefers inclusion bodies formation. While inclusion bodies give easy separation and prevent protease degradation, it has complex purification steps and refolding process is compulsory. Fusion partners, such as intein, often added in gene construction in intracellular works to provide efficient strategy in purification steps [21, 33].

Based on protein location, toxin contamination can be investigated. Both Stx and LT-ST toxins are secreted by *E. coli*, increasing the risk of contamination when the protein of interest is produced extracellularly. Even so, since extracellular protein exists in soluble state, purification might not be impossible. Whereas intracellular expression may put more concern at contamination risk since toxins might be clumped together in the form of inclusion bodies. This case may put more consideration in solubilization and purification process. However, these allegations are only an assessment of risk factors with the assumption that toxins are produced in *E. coli*, which is used for recombinant protein expression.
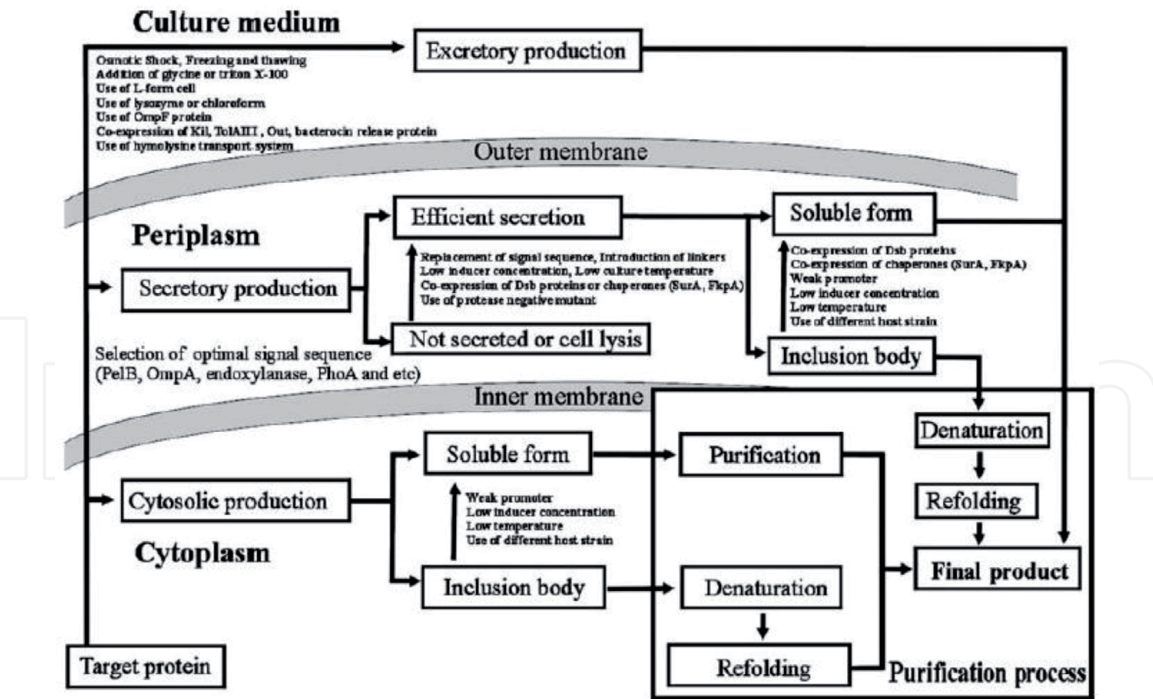
Figure 3.
Scheme of protein expressed in E. coli *and its following purification steps* [33].

## 4. Safety aspects of recombinant protein production against toxin

Using comprehensive understanding of toxin origin, specifically Shiga toxin and enterotoxin, it is clear that these toxins were peptide based and generated by certain gene in STEC and ETEC. The gene *stx* was responsible for producing the Stx toxin using central dogma of *E. coli*, reciprocally to ST and LT encoding gene. Moreover, *E. coli* strains that are commonly used for recombinant protein work are also known. Therefore, it is possible to examine the safety aspect of recombinant protein against toxin through genetic alignment between common *E. coli* strains in recombinant work and toxin genes. Here, *E. coli* BL21 (DE3) (ACC: NC_012892) and K-12 MG1655 (ACC: U00096.3) were used as representative. While toxin genes used are Stx (ACC: AY143336.1), LT (ACC: JQ031712), and ST (ACC: P22542.1).

In term of the existence of *stx* gene, common recombinant host strains are absence of the *stx* gene. Therefore, since the strains were clearly different, it is considerably safe to use *E. coli* as recombinant host without neglecting other contaminants.

5. Expression and characterization of *HSA* gene in *E. coli* BL21 (DE3)

This step started with growing *E. coli* BL21 (DE3) [pD881-torA-HSA] transformant as starter culture at 200 rpm, 37°C for 16–18 hours. Then starter culture was moved as much as 1% into 25 mL Luria-Bertani medium containing kanamycin as selection marker. *E. coli* BL21 (DE3) cell culture was grown until OD_{600nm} reached 0.8 for induction. Before induction was performed, 1 mL sample from culture was separated as protein fraction before induction (t_0). Induction was initiated by adding L-rhamnose into the expression medium to bring the final concentration to 4 mM. To obtain protein fraction in cytoplasm, sonication method was used. Lysate from six *E. coli* BL21 (DE3) [pD881-torA-HSA] transformant colonies showed that HSA was expressed in cytoplasm, it was characterized with the presence of ±67.0 kDa and in the SDS-PAGE electrophoresis [28]. The result of expression is presented in **Figure 4**.
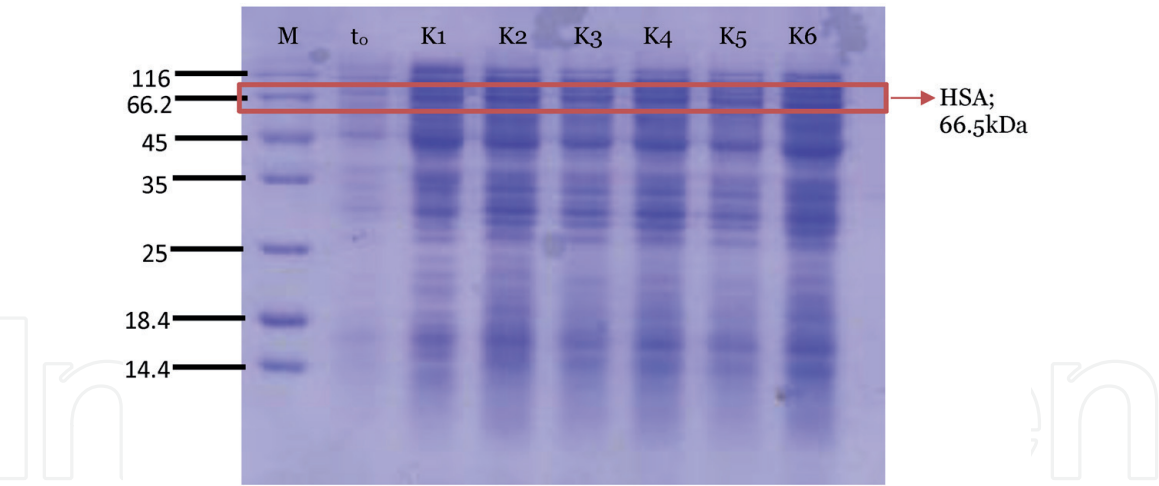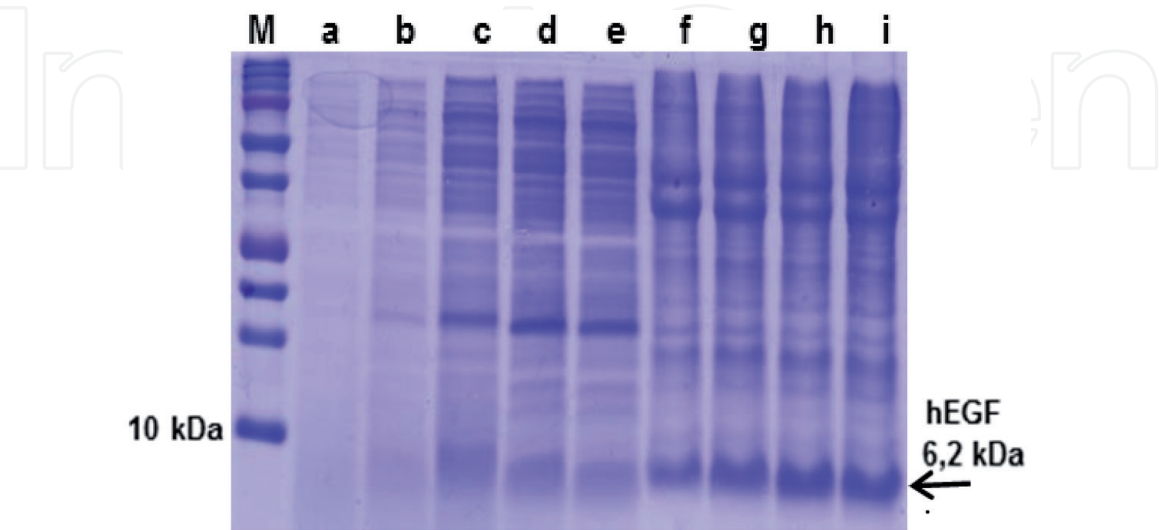
Figure 4.
SDS-PAGE electropherogram of HSA soluble fraction in cytoplasm after induction using 4 mM L-rhamnose from E. coli *BL21 (DE3) [pD881-torA-HSA] colonies. (M) Protein marker; (t_o) fraction before L-rhamnose induction; (K_i) colony transformant 1–6 after induction L-rhamnose induction [28].*

6. Recombinant hEGF expression in *E. coli* BL21 (DE3)

E. coli BL21 (DE3) [pD881-PelB] that has been characterized was grown in 5 mL LB medium containing kanamycin for about 16–18 hours at 37°C with 200 rpm shaking. Then, 1000 µL *E. coli* BL21 (DE3) [pD881-PelB] culture was transferred into 100 mL LB medium containing kanamycin in shaken flask. Native *E. coli* BL21 (DE3) was also grown and received the same treatment as the transformant. The culture was incubated until OD_{600nm} reached 0.7. *E. coli* BL21 (DE3) [pD881-PelB] culture was taken as a sample (before L-Rhamnose induction) and transferred into a microtube and centrifuged at 3000 g, 4°C for 20 minutes. L-rhamnose was added into *E. coli* BL21 (DE3) [pD881-PelB] and native *E. coli* BL21 (DE3) culture to give final concentration of 2 mM. Incubation was continued at 37°C for 20 hours with 200 rpm shaking. Culture of the *E. coli* BL21 (DE3) [pD881-PelB] was taken as a sample (after L-rhamnose induction) and transferred into 2 microtubes then was centrifuged at 3000 g, 4°C for 20 minutes [35].

Figure 5.
Electropherogram of proteins expressed by E. coli *BL21 (DE3) [pD881-PelB] with varying L-rhamnose concentration. Lane M is the protein markers. Lane (a-e) are soluble fractions of the medium with 40 µM, 1 mM, 2 mM, 4 mM, and 6 mM L-rhamnose as inducer, respectively. While lane (f-i) are inclusion bodies obtained from cell with 1 mM, 2 mM, 4 mM and 6 mM L-rhamnose as inducer. Samples were collected after 20 hours of induction [35].*

Based on SDS-PAGE electrophoresis of the protein produced by the *E. coli* cell at varying concentration of L-rhamnose as inducer (**Figure 5**), it can be concluded that the best concentration of L-rhamnose that induces the production of the protein of interest was 4 mM because it produces more target protein, either in the insoluble fraction of the medium or in the form of inclusion bodies at $t_{(20)}$. The results also indicate that not all rhEGF translocated into the periplasm were secreted to medium. The hEGF was expressed in *E. coli* BL21 (DE3) with molecular weight of 6.2 kDa. The result of expression is presented in **Figure 5** [35].

Apart from that, our group succeeded in producing beta-galactosidase from a wild type *E. coli* strain B130, with high purity. Kinetical parameter (K_m and V_{max}) of the enzyme were 2.417×10^{-4} mol and 4.664×10^{-4} mol.minute^{-1}, respectively [36].

7. Conclusions

E. coli is renowned by its pathogenic properties, specifically in causing gastrointestinal disease. While in contrast, the same species also being helpful in expressing recombinant protein. Thus, contrary properties leave questions in terms of safety in expressing recombinant protein. Pathogenic *E. coli* strains were identified and classified in accordance with the disease caused. While most of pathogenic group gain its virulence by their membrane protein, some of it secretes toxins, like Stx from STEC or LT and ST from ETEC group. This toxin-secreting *E. coli* were important to understand contamination risk in recombinant protein. All three toxins were considered as peptide-based structure, in which production relies on respective genes. Alignment of toxin genes to commonly used *E. coli* in recombinant work makes a way to investigate toxin presence in recombinant-host *E. coli*. The BL21 (DE3) and K-12 MG1655 strains used as representative in alignment process, which generate non-overlapping alignment. This clears up the risk of toxin contamination on recombinant protein since the absence of toxin gene in these strains. Therefore, expressing recombinant protein, especially therapeutic protein, in *E. coli* was considered to be safe against toxin.

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