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ORIGINAL RESEARCH PAPER

# Extracellular recombinant protein production from *Escherichia coli*

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**Abstract** *Escherichia coli* is the most commonly used host for recombinant protein production and metabolic engineering. Extracellular production of enzymes and proteins is advantageous as it could greatly reduce the complexity of a bioprocess and improve product quality. Extracellular production of proteins is necessary for metabolic engineering applications in which substrates are polymers such as lignocelluloses or xenobiotics since adequate uptake of these substrates is often an issue. The dogma that *E. coli* secretes no protein has been challenged by the recognition of both its natural ability to secrete protein in common laboratory strains and increased ability to secrete proteins in engineered cells. The very existence of this review dedicated to extracellular production is a testimony for outstanding achievements made collectively by the community in this regard. Four strategies have emerged to engineer *E. coli* cells to secrete recombinant proteins. In some cases, impressive secretion levels, several grams per liter, were reached. This secretion level is on par with other eukaryotic expression systems. Amid the optimism, it is important to recognize that significant challenges remain, especially when considering the success cannot be predicted a priori and involves much trials and errors. This review provides an overview of recent developments in engineering *E. coli* for extracellular production of recombinant proteins and an analysis of pros and cons of each strategy.

**Keywords** Autotransporter system · Kill proteins · Lysis-promoting proteins · Protein secretion · Secretion systems · Secretome

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## Introduction

*Escherichia coli* is often the first choice as host microorganism for recombinant protein production and metabolic engineering. Besides being the default organism in laboratories worldwide for expressing proteins, a significant portion of commercial therapeutic proteins is made with *E. coli*. In fact, recombinant therapeutics from *E. coli* expression systems account for nearly 40% of all marketed (Langer 2009). In applications where recombinant protein is the product of interest, extracellular production of recombinant proteins is desirable as it affords simple detection and purification, and a better folding environment free of cell-associated proteolytic degradation. Extracellular

production of toxic proteins minimizes their impact on cellular physiology and may represent the only possible way for their production. In other applications such as converting plant biomass to biofuels and in bioremediation, secretion of recombinant enzymes to extracellular milieu is crucial for the success of metabolic engineering strategy since the substrates (polymeric plant biomass or toxic pollutants) are often not adequately taken up by cells. Extracellular production of enzymes could also be useful in other areas of biological research. For example, a variant of protein generated through directed evolution and secreted into medium could be detected more easily, as compared to intracellular production that requires cell disruption and removal of cell debris (Nisole et al. 2006). This mode of production, more conducive for high throughput screening, is expected to be more useful as researchers are poised to capture vast genome information and move towards systems biology approach.

## *E. coli* naturally secretes proteins extracellularly through unknown mechanisms

*Escherichia coli* is a poor secretor of proteins. Inadequate secretion is considered one of the most significant barriers of using *E. coli* in these applications. However, it appears that *E. coli* has non-specific protein secretion mechanisms that allow mostly outer membrane and periplasmic proteins to be secreted. Indeed, several recent studies have shown low but noticeable lysis-independent secretion by some *E. coli* laboratory strains under both shake-flask and bioreactor cultivation conditions (Nandakumar et al. 2006; Rinas and Hoffmann 2004; Shin and Chen 2008), although the mechanisms by which proteins are secreted were not understood, nor were widely exploited for recombinant protein production and metabolic engineering. Proteomic analysis of the secretome showed that it consists of mainly periplasmic (70%) and outer membrane proteins but also cytoplasmic proteins (Xia et al. 2008; Nandakumar et al. 2006). The level of secretion is dependent on both strains (Xia et al. 2008) and cultivation methods. About 10 mg/l in shaker flask cultivation and 80 mg/l in high-cell-density cultivation were reported (Shin and Chen 2008; Xia et al. 2008; Nandakumar et al. 2006). Convincing evidence was shown that, in some cases, the extracellular proteins found in growth medium were lysis-independent (Nandakumar et al. 2006; Shin and Chen 2008). So far, no studies have been carried out in elucidating the mechanisms of the secretion. Vesicle formation share some similarity to the secretion, however, it is unclear at this point the role of vesicle in the secretion (Wai et al. 2003).

Accounts that a recombinant protein directed to the periplasm unexpectedly finds its way to the medium are numerous in literature. For example, clostripain, a highly specific proteolytic enzyme from *Clostridium histolyticum*, was found in growth medium when it was expressed with either its native signal sequence or the *E. coli* penicillin G acylase (PGA) signal sequence (Kim et al. 2007). Recombinant polyester-cleaving *Thermobifida fusca* hydrolase (TfH) with a *C*-terminal $His_6$-affinity tag and the OmpA secretion signal sequence was also found extracellular. Interestingly, in this case, the extracellular secretion seemed to be dependent on the cultivation methods. In batch cultivation, almost all expressed protein was exported to extracellular space whereas less than 15% of the expressed protein was found in the medium with fed-batch cultivation (Dresler et al. 2006).

Impressively, sometimes the level of secretion was significant to make it practically useful. In a high-cell-density cultivation, recombinant levan fructotransferase was accumulated in the medium to 4 g/l (Lee et al. 2001). About 60% of the extracellular levan fructotransferase retained its *N*-terminus signal peptide, suggesting it escaped the periplasm through a non-specific mechanism. In another study, extracellular recombinant asparaginase was accumulated to more than 5 g/l. Up to 75% of the expressed protein was found extracellular and the recombinant protein constituted about 40–70% of the extracellular protein depending on cultivation methods (Khushoo et al. 2005). An interesting study with recombinant pectin lyase showed that the secretion was time-dependent, the distribution of the recombinant protein among the three cellular locations (extracellular, periplasm, and cytoplasm) varied with the time. It was mostly extracellular during a short duration after the IPTG induction, subsequently it accumulated in the periplasm and, after prolonged cultivation, it finally accumulated in the cytoplasm (Papi et al. 2005).

It is not known, at this point, what mechanism is responsible for the extracellular secretion in all these cases. Circumstantial evidence points to a non-specific nature of transportation. The fact that proteins other

than recombinant proteins were also present in the medium, the incomplete cleavage of peptide sequence, the dependence on cultivation seems to be consistent with this expectation. Some kind of leakage is frequently cited. A common explanation provided by researchers is that periplasm accumulation of recombinant proteins puts stress on the outer membrane, thus the integrity of the outer membrane was compromised, leading to leakage. A similar explanation was that secretion due to compromised outer membrane stemmed from competitions of recombinant proteins with native outer membrane proteins for common translocation pathways across the inner membrane. However, no further studies providing convincing evidence were reported. Suffice it to say, however, it seems that *E. coli* does have its natural ability to secrete proteins extracellularly, across two membrane systems, albeit the level is low. The conditions that lead to recombinant protein production sometimes augment the secretion to a useful level.

## Four engineering strategies in making extracellular proteins

While the aforementioned examples were fortuitous and the extracellular secretion was often not initially intended, impressive efforts have been made by many researchers to engineer *E. coli* to produce extracellular proteins. A survey of recent literature shows that the strategies fall into four categories: (1) engineering dedicated secretion systems that naturally exist in *E. coli* pathogens; (2) use carrier proteins with no known translocation mechanisms; (3) use cell envelope mutants; (4) co-expression a lysis-promoting protein. This classification emphasizes the differences among different strategies with the understanding that they share some commonalities. The first method has a clear secretion mechanism as the basis for engineering whereas the mechanism is obscured in the second strategy, despite the fact both use fusion as ways to achieve the desired effect. The third strategy shares with the fourth in altering outer membrane permeability but they are different with respect to how to achieve the permeability change. The former focuses on engineering outer membrane components whereas the latter is achieved through expression of a lysis-promoting protein.

The review provides a concise overview of each strategy, and analyzes both the potential and limitations of each strategy. We hope the review will provide some guidance to researchers in selection of a suitable strategy for the proteins at hands, and stimulate interest in this exciting field.

### *Using dedicated secretion systems*

Six distinct secretion systems have been shown to mediate protein export through the inner and outer membranes of Gram-negative bacteria (Jermy 2009). These systems vary widely in complexity in terms of number of components involved. While type V secretion system consists of only one or two components, other systems (such as types II, III, IV) involve several dozens of proteins. To our knowledge, types I, II, III and V have been used for recombinant protein production.

*Type I* Due to its simplicity, Type I secretion system (TISS) is the most frequently used. The type I system is capable of transporting polypeptides of up to 800 kDa across the cell envelope in a few seconds. The system is illustrated in Fig. 1 with *E. coli* haemolysin (Hly) system as an example as it is the prototype and most studied Type I translocation pathway (Holland et al. 2005). As shown in Fig. 1,

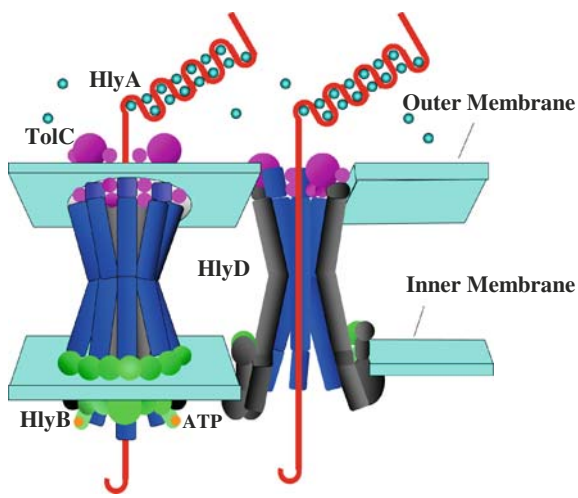


**Fig. 1** The type I secretion in *E. coli* illustrated with Hly system. Cartoon representation of TolC trimers in the outer membrane, HlyD monomers in the periplasmic space, and HlyB in the inner membrane. The interaction of these components forms a channel from the cytoplasm to the exterior, shown with a molecule of HlyA in transit. *Right*: a cutaway version of the translocator is shown. This figure is drawn following the illustration from Holland et al. (2005)

the system consists of an ABC-transporter (HlyB), a multimeric Membrane Fusion Protein (HlyD), and an outer membrane protein (TolC) (Holland et al. 2005). The substrate haemolysin (HlyA) is transported through a channel formed by the interaction of the components of the system. Therefore, the substrate is transported directly to the medium in a one-step process without forming a periplasmic intermediate. The secretion signal necessary for the recognition of the specific secretion machinery is located at the *C*-terminal end of HylA and comprises the last 46–60 amino acids of HlyA (Gentschev et al. 1996).

An early use of the TISS system to transport recombinant protein was reported in 1992. A metalloprotease from *Serratia marcescens* was successfully secreted into growth medium from *E. coli* JM101 strain carrying a plasmid that harbors HlyB and HlyD. The protease shares with hemolysin A repeating motif LXGGXGND, which may provide the necessary recognition to the secretion system (Suh and Benedik 1992). Subsequent studies demonstrated versatility of the system not only for proteins share considerable homology with HlyA but also for proteins completely unrelated. Various genes encoding proteins from *E. coli*, *Salmonella typhimurium*, *Pseudomanas* spp., *Listeria monocytogenes* and human were fused with the *C*-terminal HlyA gene, the secretion efficiency in terms of the percentage of protein expressed that are found in medium varied from only 1–2% to 90% (Gentschev et al. 1996).

Like some metalloproteases, lipase is a natural substrate of the TISS. Although *E. coli* does not naturally produce lipase, the lipase transport system from other microbes such as *Pseudomonas* was successfully used to secrete recombinant proteins from *E. coli* strains expressing the lipase TISS system. Angkawidjaja et al. investigated the secretion of a normally periplasmic alkaline phosphatase (AP) by fusing it to a *C*-terminal region of *Pseudomonas* sp. lipase (PML) in *E. coli* cells carrying a heterologous lipBCD TISS system. To study the importance of the 12 repetitive sequences to the secretion efficiency of the PML, three PML sequences with 0, 5, and 12 of repetitive sequences were designed and fused to the *C*-terminus of AP. Interestingly, all three fusion proteins were efficiently released to the medium. Only slight decrease in the secretion level with the decrease in the repetitive numbers was noted, indicating that the repetitive sequences were not critical for the secretion of the fusion protein (Angkawidjaja et al. 2006).

More recently, a thermostable lipase (TliA) of *Pseudomonas fluorescens*, featuring a typical four glycine-rich repeats in its *C*-terminus, and lipase ABC transporter domains (LARDs) of different length were studied for the secretion of recombinant proteins. The efficacies of LARDs and TliA on the protein secretion were evaluated on three model proteins including green fluorescent protein (GFP), epidermal growth factor (EGF), and cytoplasmic transduction peptide (CTP) by attaching to their *C*-termini. In *E. coli* cells carrying heterologous TISS transporter, GFP and EGF fused with LARDs or TliA were successfully exported into the extracellular medium (Chung et al. 2009).

The type I system was also exploited successfully to secrete single-chain Fv (scFv) and single-domain antibodies. These recombinant antibodies were accumulated as the sole polypeptide in the culture medium of *E. coli* culture expressing TolC/HlyB/HlyD to concentrations similar to those obtained by their periplasmic expression (0.5–2 mg/l) (Fernandez 2004).

Overall, TISS provides a versatile secretion mechanism for recombinant proteins. The level of secretion, when reported, was typically in 10 mg/l or lower. While this may be sufficient for some applications such as antigen delivery for vaccine or high throughput screening, further improvement is necessary for this to become a production method. Some more recent work aiming to improve the TISS efficiency points to the importance in balancing the rates of translation and secretion. Although the work has so far been done with HlyA, it probably will impact secretion of recombinant proteins if similar strategy (such as slowing translation rates by using rare codons) is adopted (Lee and Lee 2004; Gupta and Lee 2008). Another significant disadvantage of this method is that the product is a fusion protein, which may require post-fermentation processing steps.

*Other dedicated secretion systems* Unlike TISS, extracellular secretion via the type II secretion mechanism is a two-step process. Proteins following this pathway are synthesized as signal peptide-containing precursors and transported across the inner membrane into the periplasmic space, where the signal peptide is

cleaved and the proteins fold. In the second step, the proteins are transported outside the cell by a protein complex involving 12–16 protein components (Filloux 2004). Francetic et al. (2000) showed that the type II secretion system in common K12 strains was silenced by the nucleoid-structuring protein H-NS. In mutants lacking H-NS, the expression of *gps* genes (encoding the transport machinery) from a medium-copy-number plasmid enabled the secretion of the endogenous chitinase into the growth medium. Presumably, other recombinant type-II substrates could also be made extracellular in *E. coli* when cells are similarly engineered to express the type II system. It is well documented, however, that type II systems are less versatile than the type I systems in the sense that different components of the system from different species cannot complement each other (Lindeberg et al. 1996), limiting the use of the system. Zhou et al. demonstrated a successful use of *Erwinia chrysanthemi* out system (a type II system) to secrete an endoglucanase (celZ) from the same organism by expressing the entire Out system (40 kbp) from a plasmid in an *E. coli* B strain. Extracellular cellulase activity reached 7800 IU, representing about half of the cellulase activity and 4–6% of cellular protein (Zhou et al. 1999).

Type III translocation system (TTSS), also known as the injectisome, is a complex nanomachine of approximately 25 proteins (Mota and Cornelis 2005). The secretion apparatuses of the TTSS variants are structurally homologous but differ in substrate recognition and components of their external filaments, the needle through which the unfolded substrates are secreted (Majander et al. 2005). A modified flagellar type III secretion system was developed to secrete recombinant proteins of varying sizes (100–400 aa) with a secretion level of 1–15 mg/l (Majandar et al. 2005). The required modifications include deletion from strain MG1655 the chromosomal genes of filC and filD (encoding filament proteins). The gene of interest was fused with a 173 bp untranslated DNA fragment upstream of the gene fliC (encoding flagellin) along with a terminator from the same gene (Majander et al. 2005). This is the only example that a type III system is used to secrete recombinant proteins.

The type V secretion system includes the autotransporter family, the two-partner system and the Oca family. Similar to the type II system, the secretion is a two-step process, involving first the translocation of the precursor through the inner membrane and then its translocation through the outer membrane via a pore formed by a $\beta$-barrel (Desvaux et al. 2004). The type V secretion system is one of the most recently described pathways and the simplest system with only one component (autotransporter) and two components (the two-partner system) containing all that is required for translocation. Due to their recent discovery, the two-partner system has yet to be exploited for extracellular protein production while the autotransporter is increasingly used to display recombinant proteins unto cell surface. As the review focuses on extracellular protein production, the efforts on surface display are not detailed here. An interesting study comparing secretion efficiency with engineered heamolysin system and *Salmonella* autotransporter showed that although the expression level was similar, 30–40 ng/ml, about 88% was secreted to the medium with the haemolysin system whereas only about 6% was secreted with the autotransporter (Zhu et al. 2006).

### *Fusion protein with unknown translocation mechanisms*

The fusion protein strategy represents a useful and common means for the production of extracellular recombinant protein. Target proteins are fused to a carrier protein that is often a native extracellular or outer membrane protein (Choi et al. 2004). Recent examples of fusion protein expression for extracellular protein production in *E. coli* are shown in Table 1. This strategy differs from the first in that the mechanism by which the fusion pass through the outer membrane is unknown and host factors that influence the transport are not identified.

Early studies showed that the IgG binding domain from *Staphylococcus* protein A was a particularly good carrier. Overproduction of active and extracellular cytotoxin, α-sarcin, was achieved when fused with protein A and a signal peptide sequence, with extracellular concentration of the recombinant protein reached up to 100 mg/l (Parente et al. 1998). Protein A fusion was also successfully used for extracellular secretion of mouse-metallothinein for wastewater bioremediation (Cols et al. 2001).

YebF, a 10.8 kDa bacterial peptide, was recently demonstrated to function as an effective fusion partner that allowed recombinant protein fusions of various sizes and hydrophilicities (human interleukin-2,

**Table 1** Recent examples of fusion protein expression for extracellular protein production in *E. coli* strains

| Model proteins | Secretion proteins | *E. coli* host | Characteristics | References |
|---|---|---|---|---|
| Penicillin amidase | OmpT signal peptide | K5 | 3-fold faster in translocation rate | Ignatova et al. (2003) |
| α-Amylase | YebF | HB101 | 42.6% of protein secreted to the medium | Zhang et al. (2005) |
| *Thermobifida fusca* hydrolase (TfH) | OmpA | TG1 | Activity of 8 U/ml in the medium, almost 100% secreted | Dresler et al. (2006) |
| Alkaline phosphatase (AP) | *C*-Terminal region of *Pseudomonas* sp. MIS38 lipase (PML) | DH5 carrying Lip system | Activity of 10 mg/l in the medium | Angkawidjaja et al. (2006) |
| Clostripain | Penicillin G acylase (PGA) signal sequence | BL21(DE3) | Activity of 405 U/l in the medium, 80% secreted | Kim et al. (2007) |
| Polyhydroxybutyrate depolymerase (PhaZ1) | Signal peptide of PhaZ1 and anchoring motif INPNC | BL21(DE3) | Activity of 3.47 U/l in the medium, 60% secreted | Park et al. (2008) |
| Green fluorescent protein (GFP), epidermal growth factor (EGF), and cytoplasmic transduction peptide (CTP) | Lipase ABC transporter domains (LARDs) or whole thermostable lipase (TliA) | XL1-Blue carrying ABC transporter | Evident secretion of fusion proteins into the medium | Chung et al. (2009) |

α-amylase and alkaline phosphatase) to be secreted extracellularly from *E. coli*. In particular, about half of YebF-α-amylase fusion was secreted into the medium. Importantly, the expression of YebF did not affect the cell membrane sensitivity towards antibiotics, detergents, and lysozyme. Although the mechanism of the YebF secretion is not fully understood, a two-step process involving transporting across the inner membrane through a sec-dependent system followed by exporting to medium via an unknown system on the outer membrane was proposed (Zhang et al. 2006).

In another study, Park et al. (2008) reported the development of a useful expression and secretion system for *Paucimonas lemoignei* polyhydroxybutyrate depolymerase (PhaZ1) in *E. coli* with the aid of an outer membrane protein, ice nucleation protein (INP). In addition to the signal peptide of PhaZ1, the extensively studied Gram-negative bacterium surface display protein INP, was fused to PhaZ1 in its truncated form (INPNC) to enable the secretion of active PhaZ1-fusion into the medium. Besides its role in protein translocation, INPNC was capable of alleviating the toxicity effect of PhaZ1 on cell growth and plasmid stability. The fusion protein was predominantly located in the extracellular medium (60%) under cultivation temperature of 37°C.

ClyA is a cryptic hemolysin found in *Salmonella enterica* serovar Typhi. Its *E. coli* homolog is secreted by a mechanism involving outer membrane vesicles (Wai et al. 2003). When ClyA was used as fusion partner, several antigens, including an anthrax-protective antigen, were successfully secreted from a recombinant strain (Galen et al. 2004). Although the work was done with *Salmonella*, presumably it (or its *E. coli* homolog) could be similarly used for secretion of recombinant proteins from *E. coli* strains. It would be interesting to see whether the fusion follows the vesicle-mediated secretion.

Although the exact mechanism of translocation through the outer membrane for many fusion proteins is largely unknown, this strategy is often quite effective. The use of the right partner is important in determining the efficiency of secretion. This motivates researchers to look for more effective fusion partners. Qian et al. (2008) applied proteomic analysis to extracellular proteins from *E. coli* high-cell-density culture. From prominent protein spots with molecular weights below 40 kDa, 20 were

selected as candidate fusion partners, 12 of which allowed fused proteins to be secreted into the medium in considerable amounts. The best partner, OsmY, was used to fuse with three recombinant proteins and the secretion level was from 5 to 64 mg/l in shake-flask cultures. Using a carrier protein is a versatile; method; however, the disadvantage is the need to cleave fusions in order to obtain authentic proteins. In addition, unless the fusion partner is small, the size of the fusion could become a limiting factor, as large proteins are more difficult to pass though the membrane in general.

*Using cell envelope mutants*

Mutants with defects in outer membrane structures were exploited as host for extracellular protein production. This approach necessitates an intermediate accumulation of proteins in the periplasmic space, which is usually accomplished by fusing an *N*-terminal sequence to the gene of interest and most commonly, sec-dependent or TAT-dependent pathways are used for the translocation of fusion proteins through inner membrane. In the second step, recombinant proteins in the periplasm escape into the medium due to the increased permeability from defects in outer membrane structure. In one extreme case, wall-less *E. coli* strain or so-called L-forms were used, although due to extreme fragility of cells and sensitivity to environmental stresses typical of a larger fermentor, this type of mutant may be of limited use for large-scale applications (Gumpert and Hoischen 1998). One important advantage of the approach is its non-specificity, accommodating a wide range of proteins that are engineered to express in periplasm. Therefore, no protein specific engineering is required. A potential disadvantage is the effect of membrane mutations on cell growth. However, recent studies from authors' laboratories suggest that the effect on cell growth may not necessarily be limiting so long as the target of the mutation is carefully selected. Interestingly, a single *lpp* gene deletion significantly increased the outer membrane permeability without eliciting cell lysis. In fact, the *lpp* mutant showed an identical growth rate compared to the wild type. The only difference in growth was found toward the end of the cultivation. The final cell density was about 20% lower than the control strain without deletion (Ni et al. 2007). High-percentage secretion of recombinant proteins to extracellular medium was shown for three model proteins, maltose-binding protein (MalE), a xylanase and a cellulase. As shown in Fig. 2, up to 90% of the recombinant xylanase activity was found in growth medium with the deletion mutant whereas only about 40–50% was secreted with the control strain (Shin and Chen 2008) when cells were grown in a bioreactor under identical conditions. The extracellular secretion in the mutant was observed throughout all growth phases including the exponential phase, suggesting a lysis-independent mechanism. Further testing with cytoplasmic enzyme activity confirmed that secretion was not due to lysis (Shin and Chen 2008). Clearly, this example shows that the natural secretion could be augmented by judicious membrane engineering.

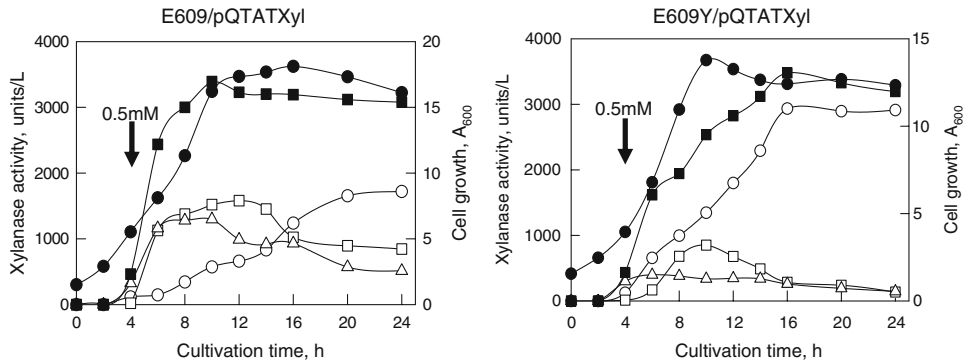


**Fig. 2** Bioreactor cultivation of *E. coli* E609/pQTATXyl and E609Y/pQTATXyl for xylanase production. *Symbols*: *filled circle*, cell growth; *open circle*, extracellular xylanase activity; *open triangle*, periplasmic xylanase activity; *open square*, cytoplasmic xylanase activity; *filled square*, total xylanase activity. The *arrow* shows the point of IPTG induction

*Co-expression of lysis-promoting proteins*

The expression of *kil* gene (kill protein) for extracellular secretion was first reported by Robbens et al. (1995) and was further studied for the secretive production of several prokaryotic and eukaryotic proteins, including bacterial phytase and murine interleukin-2 (Choi and Lee 2004). The *kil* gene, encoding a 6 kDa protein, is modified by addition of a fatty acid followed by splitting into two peptides. The expression of *kil* was shown to alter permeability in the outer cell membrane, and as a result, the periplasmic proteins were released into the medium (Suit et al. 1998).

The effect of Kil coexpression on *E. coli* periplasmic penicillin amidase (PA) was investigated by Ignatova and coworkers. Significant increase in both extracellular and total PA activity was noticed after 6 h of induction (Ignatova et al. 2003). Since the expression of *kil* could affect the viability of the cells, the induction time and the promoter strength for Kil expression are crucial. Using *Bacillus* β-glucanase as a model protein, Beshay et al. (2007) investigated four expression vectors harboring all possible combinations of a weak and a strong stationary-phase promoter for the *kil* and β-glucanase genes, respectively. Despite of the potential negative influence of *kil* gene on the cell growth, higher extracellular β-glucanase was reached when the expression of β-glucanase was regulated by a strong promoter, in which over 90% of the total β-glucanase was secreted into the medium. The positive effect of the strong promoter was attributed to the prolonged secretion phase. The study indicated that the extracellular production of recombinant protein could be improved by tuning the promoter strength of each protein components. Recently, they demonstrated the simultaneous production and recovery of extracellular β-glucanase through co-expression of *kil* and metal-chelating affinity chromatography in a bioreactor equipped with special separation device containing charged PDC resins (Beshay et al. 2009).

In another study, Miksch et al. (2008) reported optimization of promoter strength for the *kil* gene for the extracellular expression of streptavidin (SA). A strong stationary-phase promoter for *kil* expression resulted in better SA expression and its extracellular secretion than using weak promoters. In addition, *phoA* leader sequence, and supplementation of glycine in the culture medium had positive effects on the secretion, suggesting their synergistic effect on the extracellular yield of recombinant protein.

Similar to Kil, a bacteriocin release (BRP) protein, also known as 'lysis protein', was used for extracellular protein production. BRP is a small lipoprotein of 28 amino acids, when expressed at high-level, it leads to quasi-lysis and lethality (Rahman et al. 2005). Several proteins, such as β-lactamase, human immunoglobulin, penicillinase, penicillin acylase, F1 alkaline protease, and lipase, were secreted with the co-expression of BRP (Kitai et al. 1988; Lurink et al. 1987; Jin et al. 2001; Fu et al. 2003; Rahman et al. 2005). Under optimized condition, at a rather low IPTG concentration (0.05 mM), presumably to reduce the effect of BRP on cell viability, the secretion of 18,100 U/ml thermostable T1 lipase was reported, demonstrating the effectiveness of the secretion through this method (Rahman et al. 2005).

This method, due to its non-specificity, is potentially useful for a wide range of proteins. The disadvantage of the strategy is the need to optimize the expression of the lysis-promoting protein, relative to the desired product since high expression of Kil or BRP have detrimental effects on cells leading to cell lysis. Additionally, the presence of other cellular proteins due to leakage of periplasmic proteins, which, depending on applications, may necessitate additional steps for their removal, although the task of removal is made much easier as compared to cytoplasmic expressions where all cellular proteins are contaminants in cell lysate.

## Prospective

Over the past decade, significant progress has been made in engineering *E. coli* to secrete proteins into growth medium. Both specific and non-specific translocation mechanisms could be used as basis for engineering. Most remarkably, the level of secretion reached several grams per liter in several cases, which places *E. coli* on par with common eukaryote systems in the secretion capacity. While these results are certainly encouraging, it is important to note the challenges that remain. The highest secretion was observed with proteins directed to periplasm, the exact mechanism through which the proteins cross the outer membrane remains unknown. This makes rational engineering impossible and the success relies, on a

large part, on trials and errors. Little is known about how secretion efficiency is influenced by the nature of proteins or protein fusions (size, 3-D conformations etc.). If the secretion is stress related, what is the trigger and how to exploit it without making cell lyse? Even with mechanisms known in the case with dedicated secretion systems, there are still significant challenges. For product concentration, one or two orders of magnitude of improvement are needed (mg to g/l) for most applications. Even when all other factors equal, we frequently observe different proteins secrete with very different efficiencies. What are the molecular determinants for secretion? Could we make the process more predictable? These questions await researchers in the field. Nevertheless, we are confident that with the advent of various powerful systems biology tools, the use of *E. coli* for extracellular protein production will be advanced at a more rapid pace than witnessed in the last decade. More rationality will become apparent as to predictability for outcomes, providing crucial guidance on selection of the right host background and right secretion mechanism to match the properties of the protein of interest.

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