Parameters Influencing the Productivity of Recombinant *E. coli* Cultivations

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Dedicated to Professor Dr. Karl Schügerl on the occasion of his 65th birthday

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In the past 10 to 15 years, many of the promises of microbial genetic engineering have been realized: the use of recombinant *Escherichia coli* has moved from the laboratory to the production facility, and the manufacture of therapeutic recombinant proteins such as human growth hormone and interleukins is a rapidly growing industry.

Along with this progress, however, have come new problems to solve: bioreactor operators have discovered that large-scale cultivations of plasmid-containing bacteria do not behave in exactly the same way as those of plasmid-free cells, plasmid stability has been recognized as a major hurdle, and the protein product might not be present in a soluble form but rather as intracellular granules that resist solubilization. These and other difficulties represent a new generation of challenges for genetic engineering.

However, genetic engineering can do more than solve these problems. Molecular biological techniques also have the ability to create new opportunities: to produce new compounds, to use cheaper substrates, to facilitate downstream processing, and to optimize production in new ways.

The productivity of a cultivation can generally be expressed as the product of the cell density and the specific biological activity. Both of these parameters are influenced by a variety of factors. For recombinant cultivations, though, the level of biological activity, a reflection of the plasmid copy number and expression efficiency, is the more interesting and important consideration and will therefore be given more attention in our review. In this contribution, our general goal is to discuss the factors that influence the productivity of recombinant *E. coli* cultivations, covering

- parameters relating to DNA;
- parameters relating to protein synthesis;
- parameters relating to proteins; and
- parameters relating to downstream processing.

The object is not to tell the reader how to choose the perfect plasmid, host, and cultivation conditions, but to make known the many variables involved in designing a recombinant process and to point out recent and potential advances made possible by genetic engineering. The discussion focuses on the production of a protein, but many of the same concepts apply to other cultivations of recombinant *E. coli*, including cases in which the desired product is not a protein or the cells have been designed for a special metabolic capability such as pollutant biodegradation.

1 Parameters Relating to DNA

1.1 Plasmid Copy Number

It is often true that an increase in the number of copies of a gene (the "gene dosage") will result in an increase in the production of that gene's product protein (the "gene dosage effect"). While this is not always the case – regulatory mechanisms or a saturation effect may impose an upper limit [1] – increasing the copy number of a plasmid is a common method to enhance the productivity of a cultivation.

1.1.1 Increased Copy Number by Plasmid Design

Since the copy number is closely related to plasmid replication, it has been possible to design higher copy number plasmids by modifying replication functions. Examples of this include:

- *cop* and *rom* mutations (in ColE1 derivatives)
- Multiple tandem gene repeats
- Runaway replication plasmids
- Dual low/high copy number origins of replication

The first two of these provide a high plasmid copy number throughout a cultivation. The *cop* and *rom* mutations in ColE1-type plasmids influence the regulation of plasmid replication [2, 3]. These types of mutations have been used to produce ColE1 derivatives maintained at a level of 500 copies per cell [4]. Similar opportunities for exploiting mutations in replication regulation are available for plasmids such as R1, R6K, and pSC101 [5, 6, 7]. The use of tandem gene copies in a plasmid has also been successful; for example, *E. coli* that contained a plasmid with four copies of the chloramphenicol acetyltransferase gene produced four times as much product as cells harboring a plasmid with a single gene copy [8]. However, tandem gene copies are prone to homologous recombination.

Since continuous maintenance of plasmids at high copy numbers is a burden to the host cell, resulting in significantly reduced growth rates as well as increased plasmid instability, plasmid designs allowing one to increase the copy number at an appropriate point in a cultivation are often desirable. One such design is a temperature-sensitive "runaway" replication mutant, with which the copy number of a plasmid is low at 25 °C but rapidly increases when the temperature is shifted to 37 °C [9]. A different method involves the use of both high and low copy number origins of replication on the same plasmid. Here, the idea is to insert a controllable promoter in front of the replication primer of the high copy number plasmid. An example of this is pMG411, which is maintained at 4 copies per cell at 30 °C and at 140 per cell when the culture temperature is increased to 42 °C [10].

1.1.2 Influence of Cultivation Conditions

Cultivation parameters also play a role in determining the copy number; thus, it is also possible to change the copy number of a plasmid by manipulating the cultivation conditions. The effects vary with the plasmid being used, but some trends are evident.

Seo and Bailey [11] utilized different media to change the growth rate (and perhaps other influential parameters) in batch cultures, and observed that the copy number increased with decreasing growth rate. The influences of nutrient limitation in continuous culture have also been studied. When a minimal medium was used in a chemostat with glucose as the limiting substrate, Jones et al. [12] noted significant decreases in copy number after one week of operation.

When using runaway replication plasmids, the culture conditions have a high impact on the achievable copy number. For example, the influence of substrate feeding during runaway induction of pOU140 is shown in Fig. 1 [13]. Clearly, the addition of the carbon source, lactose, led to a significantly greater number of plasmid copies than in the case in which no substrate was added.

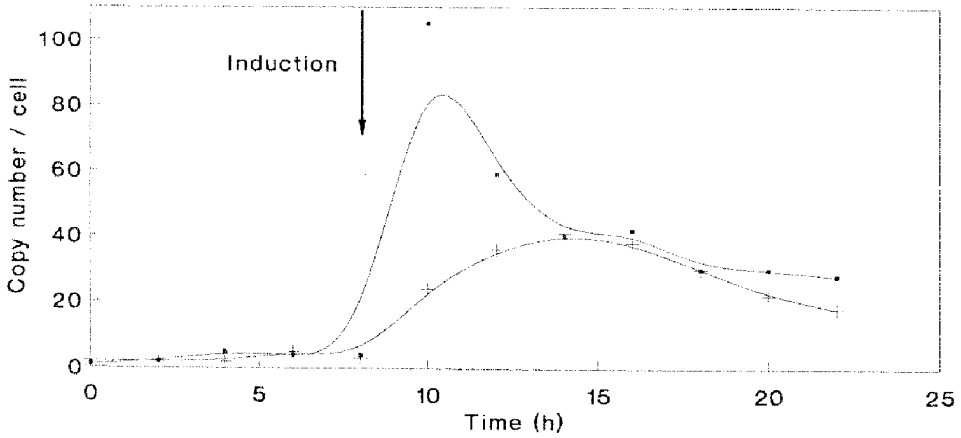

Fig. 1. Runaway replication with addition of lactose medium at point of induction. (■ with addition; + without addition)

1.2 Plasmid Stability

One of the most important issues affecting the productivity of recombinant *E. coli* cultivations is the maintenance of plasmids within the host cells. Plasmid instability is of two types: segregational (plasmid loss resulting from defective partitioning during cell division) and structural (undesired plasmid modifications resulting from insertion, deletion, or rearrangement of DNA).

Factors influencing both types of instability and strategies for overcoming these problems will be discussed in the following sections.

1.2.1 Segregational Genetic Stability

Although some wild type, low copy number plasmids exhibit high stability, most plasmids of industrial interest (high copy number and expressing heterologous proteins) are lost at frequencies of 10^{-2} to 10^{-5} per cell per generation [14]. A recombinant host-vector system with an appreciable level of segregational plasmid instability will be less productive than desired for two reasons:

- the lowered average copy number will generally result in lower specific productivity; and
- plasmid-free cells which eventually emerge have a higher specific growth rate (since they are no longer metabolically burdened by the plasmids) and will thus reach a higher concentration than the plasmid-containing cells.

In a model developed by Imanaka and Aiba [15], cells were grouped as either plasmid-containing or plasmid-free (i.e. copy number was not considered). Two parameters were used to determine the fraction F of plasmid-containing cells remaining after N generations: the frequency of plasmid loss (formation of plasmid-free cells) and the ratio of the growth rates of plasmid-free to plasmid-containing cells. The value of F was much more sensitive to the growth rate ratio than to the loss frequency.

Strategies for overcoming segregational plasmid instability are based on the alteration of one of these key parameters. Several types of approaches can be identified. These have been classified as either selective (based on eliminating plasmid-free cells) vs non-selective [16], or cellular/molecular vs bioprocess methods [17]. In the following paragraphs, methods for preventing or overcoming plasmid instability problems will be discussed according to the level at which they are implemented, i.e.

- plasmid construction
- plasmid copy number
- cultivation conditions
- bioreactor configuration

Influences of plasmid construction. The composition of a plasmid influences its stability and thus provides the basis for the most common approaches for enhancing plasmid maintenance. Perhaps the best known of these is the inclusion of an antibiotic-resistance gene on the plasmid combined with the addition of that antibiotic to the medium. Although this method is simple to implement, it has a number of disadvantages on an industrial scale, including the cost of the antibiotic and the need to separate the antibiotic from the desired product.

In another selective approach, a gene essential to the host cell (usually a mutant) is included in the plasmid. Examples of this are the *serB* gene (for serine production) in a *serB*$^-$ host [18], the *valS* gene (encoding valyl tRNA-synthetase) in a *valS*^{ts} host [19], and the *ssb* gene (for the SSB protein) in a *ssb*$^-$ host [20]. This method has been very successful; for instance, plasmid stability was

maintained for more than 200 generations in the *valS* system [19]. However, problems can arise when the mutated host cell is unable to grow rapidly or when spontaneous reverse mutations occur.

Selection for plasmid-containing cells can also be accomplished by killing plasmid-free cells. In one approach, the *parB* locus of plasmid R1 is incorporated into the plasmid. This sequence includes two genes: *hok*, which produces a potent bacteriocin, and *sok*, which forms an mRNA product that prevents the translation of *hok* mRNA. Since the *sok* mRNA has a much shorter lifetime than *hok* mRNA, cells that lose the plasmid after division are killed by the bacteriocin [21]. An alternative method of killing plasmid-free cells is to include the bacteriophage λ repressor on the plasmid and to infect the host cells with the phage. Any plasmid-free cells that appear are then lysed [22].

The use of adjustable copy number (runaway replication) plasmids is a different type of method that allows both good plasmid stability and high productivity. Plasmid stability is maintained during an initial low copy number growth phase due to a favorable ratio of growth rates between plasmid-containing and plasmid-free cells. Following the growth phase, the copy number is increased (e.g. by a temperature shift) and a period of high rates of product formation occurs [9]. This approach can be applied to continuous cultivation by utilizing a two-stage chemostat [23].

Most of the preceding methods are similar in that plasmid-containing cells are given a growth advantage over plasmid-free cells without inherently increasing the segregational plasmid stability. Another problem with selective techniques is that they do nothing to maintain plasmid copy number but rather require only that one plasmid per cell be present.

Non-selective, or genetic, approaches are more promising for ensuring that high copy numbers are stably maintained. An example of these methods is the inclusion of strong terminator sequences on the plasmid to prevent stability problems posed by the strong promoters that are frequently used [24].

The *par* locus of pSC101 is responsible for stable maintenance of that plasmid. When *par* has been cloned into other plasmids, their loss frequencies have decreased [25, 26]. Meacock and Cohen [26] determined that the *par* locus effectively increases the stability of low copy number plasmids but had little impact on high copy number vectors. It should also be noted that insertion of the *par* sequence can reduce the copy number [27].

Another example of a genetic method for enhanced plasmid stability is the incorporation of the *cer* locus of the plasmid ColE1. When plasmids containing *cer* are grown in a *xer*$^+$ (genes for site-specific recombination) host, multimerization of plasmids is reduced, leading to increased plasmid stability [14].

Influences of plasmid copy number. The number of plasmids per cell has an influence on the segregational plasmid stability of the culture. Although low copy number plasmids with an active partition mechanism (e.g. *par*) are usually very stable, high copy numbers generally result in greater stability when random

partitioning occurs at cell division [2]. However, this stability advantage of a high copy number system can be offset by the decreased growth rate associated with such cultures, so that the generation of even a few plasmid-free cells can lead to domination by the faster-growing segregant.

Influences of cultivation conditions. The growth environment of a recombinant cell can have a significant effect on the segregational stability of the plasmids it carries. Variations of plasmid stability with differences in growth rate, medium composition, dissolved oxygen concentration, and temperature have been observed; however, it has been difficult to find real trends in most cases.

Plasmid instability has generally been observed to increase with decreasing growth rate, primarily because the relative growth rate advantage of plasmid-free cells over those containing plasmids is decreased under these conditions [23].

The influences of medium composition are less clear, due in part to differences between host strains. Nutrient effects are greater in chemostat cultivations, where at least one nutrient is limiting, than in batch cultures [28]. Although exceptions have been reported, continuous cultures limited by phosphate and magnesium generally exhibit high plasmid instability [12]. Instability under glucose limitation is often lower [29, 30], and nitrogen limitation does not appear to affect plasmid maintenance [29]. Plasmid instability can often be lessened with the addition of complex nutrients like casamino acids [31].

Limiting levels of dissolved oxygen have a detrimental effect on plasmid stability; both short-term (oxygen shock) and long-term oxygen limitations have been shown to increase the rate of plasmid loss [32, 33]. Finally, plasmid stability is generally found to decrease with increased temperature [34].

Bioreactor configuration. Several novel bioreactor designs have been proposed to increase the plasmid stability of a culture. Such alternatives to traditional batch or chemostat cultivations include two-stage chemostats (e.g. for use with runaway replication vectors [23]) and cycling of growth conditions between different dilution rates [35], substrate concentrations [36], and temperatures [37]. A special cell-recycle reactor has been used to maintain high levels of plasmid-containing cells by taking advantage of a flocculation sequence on the plasmid [38]. Cell immobilization has also been shown to increase the plasmid stability of a culture [39].

1.2.2 Structural Genetic Stability

Structural plasmid instability can be difficult to detect in a cultivation, since the growth rate and marker phenotype are the same as those of the desired cells. Several studies have shown that structural instability is affected by the cell's environment; for example, Godwin and Slater found different types of structural changes in glucose- and phosphate-limited chemostats [40].

2 Parameters Relating to Protein Synthesis

2.1 Promoters

The synthesis of a protein starts with the promoter. The initiation of transcription is a rate-limiting process for mRNA synthesis. Comparisons of more than 100 promoters of *E. coli* have shown that there are two regions of conserved DNA sequences [41, 42]. These regions, located 10 and 35 base pairs upstream from the transcription initiation site, strongly influence the strength of a promoter, which in turn determines the rate of transcription initiation. During the development of the molecular biology of *E. coli*, many different promoters were investigated; some of them are currently used in biotechnology and others may be useful after further study.

2.1.1 Promoter Strength

Due to the importance of the promoter strength on the productivity of a recombinant cultivation, genetic engineering is widely used to enhance the strength. The sequence of well known promoters such as *lacUV5* have been changed and the effects on promoter strength examined [43]. New sequences are often tested in order to find especially strong promoters like λP_L and λP_R from the bacteriophage lambda.

While it is important to use strong promoters in the production of recombinant proteins, regulation of those promoters is essential since constitutive overproduction of heterologous proteins leads to decreases in growth rate, plasmid stability, and culture viability. Some promoters are regulated by the interaction of a repressor protein with the operator (a region downstream from the promoter). The most well known operators are those from the *lac* operon and from bacteriophage λ. An overview of regulated promoters in *E. coli* is presented in Table 1.

2.1.2 Induction

A major difference between typical bacterial cultivations and those involving recombinant *E. coli* is the technique of separating growth and production phases. This method takes advantage of regulated promoters to achieve high cell densities in the first phase (while the promoter is "off" and the metabolic burden on the host cell is slight) and then high rates of heterologous protein production in the second phase (following induction to turn the promoter "on").

For industrial bioprocesses, low-cost induction systems are desirable. This is still a problem; for example, the widely used *lac* promoter system is induced with IPTG, a relatively expensive compound. Another common induction technique

Table 1. Regulated promoters in *E. coli*

Induction	Promoter	Operator	Ref.
Temperature shift	λ_{PL}	λ	44
	λ_{PR}	λ	45
	λ tandem	λ	46
IPTG	lac	lac	47
	tac	lac	48
	lac mutations	lac	49
	lpp trp tandem	lac	50
	mac	lac	51
	rac	lac	52
	rrnBP2	lac	53
	Synthetic consensus	lac	54
	T7 gene 10	lac	55
IAA	trp	trp	56
	lpp trp tandem	trp	50
Arsenite	ars	ars	57
Dissolved oxygen	trp	trp	58
	vgb	vgb	59
CO_2 limitation	?	?	60
Fe limitation	?	?	60
Mg limitation	?	?	60
NO_3^{2+} limitation	?	?	61
PO_4^{3+} limitation	ugp	ugp	62
	psi	network	63
N limitation	glnHP2	glnHP2	64
pH shift	alx	alx	65
	λ_{PL}	?	66
Osmotic pressure	bet	?	67
	otsA	?	67
	otsB	?	67
	proU	proU	68
	treA	?	67
Redox potential shift	?	?	69
Succinate	tna	tna	70
Deoxyribose phosphate	deo P1P2	?	71
SOS response	λ_{PL}	?	72
Tryptophan limitation	trp	trp	73

used with promoters like λP_L is a temperature shift, which requires large amounts of energy and may also lead to the undesired formation of stress response proteins. Induction by a pH shift or by addition of an inexpensive inducer molecule would be of great biotechnological interest.

2.2 Terminators

The first step of protein biosynthesis (mRNA formation) ends with the termination of DNA transcription, when the RNA polymerase reaches the terminator sequence. Without correct termination, the mRNA would form the wrong proteins, leading to an overall decrease in productivity. The use of strong

promoters requires strong terminators to prevent transcription readthrough. A variety of transcription terminators have been utilized, including the bacteriophage fd terminator [74], and some are commercially available (e.g. the *trpA* terminator) [54].

2.3 mRNA

The first product of protein biosynthesis is mRNA, which is then translated to form the protein of interest. In addition to structural factors of the mRNA that influence its translation, the amount of translatable mRNA has a direct impact on the overall productivity. The amount of mRNA in a cell is a function of the rate of transcription (discussed above) and the rate of mRNA degradation. This degradation rate depends on the presence of RNase recognition sequences, especially those for 3′-exonucleases. Chan et al. have tried to increase the halflife of mRNA by using non-essential regions of an intron [75]. This research led to the development of the cloning vector pKTN-CAT, which increased the production of chloramphenicol acetyltransferase three- to seven-fold by stabilizing the mRNA molecules [76].

A second method to increase the lifetime of mRNA involves the product of the *ams* (altered mRNA stability) gene. Strains that carry the temperature sensitive *ams-1* mutation have longer mRNA halflives at the non-permissive temperature [77].

Another possibility for enhancing the halflife of mRNA is to use RNase-deficient mutants [78]. However, such mutants are often difficult to cultivate.

2.4 Ribosomal Binding Sites

The translation of mRNA, the second step in protein biosynthesis, starts with the binding of the ribosomes at specific binding sites (RBS) on the mRNA molecule. Weak binding sites lead to a low level of expression. In many systems, the expression of foreign genes utilizes the native RBS. An increase in the expression level can be achieved by replacing this natural RBS with an altered, more efficient sequence [79]. For example, Olsen et al. were able to enhance the expression of bovine growth hormone in *E. coli* by enriching the sequence flanking the RBS with A and T nucleotides [80]. The distance between the RBS and the AUG start codon also has an impact on the rate of initiation of translation [81]. A more complete understanding of the influence of the secondary structure of the RBS on the rate of translation should provide further opportunities to optimize foreign gene expression [82].

2.5 Stop Codons

As in the case of transcription, translation also requires an efficient termination sequence. These translation stop signals have been added to commonly used vectors like pUC12 (forming pUC12-STOP) [83]. This vector was constructed by inserting a DNA linker with TAA translational stop codons in all three reading frames. Other terminators, such as the Universal Translation Terminator [54], are commercially available.

2.6 Codon Usage

When using synthetic genes, the amino acid sequence of a protein is used to develop a DNA sequence. Due to the nature of the genetic code, a choice of codons is usually available. It has been shown that the use of particular codons can play a role in gene expression. For example, highly expressed genes in several species show a bias for certain synonymous codons [84]. Differences in codon usage can also influence mRNA lifetimes.

3 Parameters Relating to Proteins

3.1 Proteolysis

Heterologous proteins produced by *E. coli* are usually subject to attack by a variety of proteases. *E. coli* cells produce cytoplasmic, membrane-bound, and periplasmic endoproteases. Relatively little is known about their induction, substrate sequences, or kinetics.

Several methods have been devised to minimize proteolytic activity on the cloned gene product. These include the use of low protease hosts, inhibition of proteases, disguising the desired product by forming a fusion protein, excreting or secreting the product to a "safer" location, and overwhelming the proteolytic enzymes with a high rate of product formation. Modification of cultivation conditions can also reduce protease levels; for example, some proteases are synthesized in response to low dissolved oxygen or glucose conditions.

3.1.1 Protease Deficient Strains

The levels of protease activity vary among *E. coli* strains, and this may represent one criterion for host strain selection. More directed efforts have focused on mutants deficient in the production of one or more of the known proteases. The best known examples are the *lon*$^-$ mutants, which cannot form the cytoplasmic

protease La [85]. Although cloned protein accumulation can be higher in *lon*$^-$ hosts [86], removal of one protease does not eliminate proteolytic activity. Drawbacks to the use of protease mutant hosts include their altered physiology; for example, *lon*$^-$ strains are UV sensitive and some exhibit a mucoid phenotype that makes cultivation problematic.

3.1.2 Inhibition of Proteases

Although it is more difficult to inhibit intracellular proteases than those present in the cultivation medium, at least one such effort has been successful. In this case, the *pin* sequence (encoding a protease inhibitor produced during bacteriophage T4 infection) was cloned onto a plasmid, effectively reducing the degradation of the desired product [87].

3.1.3 Fusion Proteins

It is often possible to protect a heterologous protein from proteolysis by producing it as a hybrid product with a native protein such as β-lactamase [88] or special foreign proteins like ubiquitin [89]. The use of fused sequences can also enhance translation initiation, and the hybrid products can be designed to facilitate purification. However, the cleavage of the fused molecule to obtain the desired product requires additional processing and may be difficult. Also, expression of a fused protein may be lower than expected.

3.1.4 Protein Export

Another method to reduce proteolysis of the product protein is to engineer its excretion from the cytoplasm into the periplasmic space or its secretion into the medium. Transport into the periplasmic space can be accomplished by fusing leader peptides for periplasmic or outer membrane proteins to the product protein. Although the process of directed transport is not well understood and selection of the best leader sequence is done by trial and error, this approach has proven successful in many cases (e.g. [90]). Additional aspects of protein secretion and excretion are presented in Sect. 4.2.

3.1.5 Rapid Product Formation

Finally, producing the cloned gene product at high rates so as to saturate the proteolytic enzymes is an effective method of minimizing degradation. This can be accomplished with runaway replication vectors or with plasmids containing inducible promoters. Since this strategy has other benefits (e.g. increased plasmid stability), it is frequently implemented.

Rapid production of heterologous proteins often results in the formation of insoluble inclusion bodies. Although these protein aggregates offer further protection from proteases, recovering active product molecules can be problematic.

3.2 Inclusion Bodies

In *E. coli* (and other microorganisms), the product of a foreign gene frequently appears in the form of proteinaceous aggregates called "inclusion bodies" or "refractile bodies". The desired protein can make up from 40 to 95% of the total protein content of these particles; contaminants include other proteins (especially outer membrane proteins), lipopolysaccharides, and membrane fragments [91, 92]. Inclusion bodies can be found in the cytoplasm or in the periplasmic space [93].

The implications of inclusion body formation are mixed for cultivations of recombinant *E. coli* in which a protein is the desired product. (Of course, when the foreign protein is not the desired product, formation of inclusion bodies is detrimental.) Although total protein productivity may be enhanced, the process economics may be adversely affected. These tradeoffs, as well as the factors influencing inclusion body formation, are discussed in the following paragraphs.

3.2.1 Advantages and Disadvantages of Inclusion Body Formation

The production of inclusion bodies can enhance protein production in recombinant *E. coli* cultivations in two areas. First, protein accumulation often increases, primarily because proteins in an inclusion body are resistant to protease attack. The formation of inclusion bodies can also allow the production of high levels of proteins that are toxic to the host cell when soluble.

Due to their physical characteristics, inclusion bodies also offer the opportunity to improve recovery of the protein product from a cultivation. A high degree of purification can be achieved by cell lysis and simple low-speed centrifugation. Since proteins in inclusion bodies are already denatured, additional purification steps can utilize conditions (e.g. detergents) and techniques (e.g. gel filtration chromatography) that are less effective or inappropriate for active proteins.

However, protein production in the form of inclusion bodies can also have significant disadvantages for the bioprocess. One problem involves the release of endotoxins when cells are disrupted to free the aggregates. This is a concern for the purification of any intracellular protein and can only be avoided by secreting the product into the medium.

The larger difficulty posed by inclusion bodies is the renaturation of the protein to obtain an active product; in addition to the technical problems inherent in this task, the process economics can be negatively affected by the special renaturation steps. Following solubilization of the protein particles with

concentrated solutions of urea, organic solvents, and/or chaotropic salts (e.g. guanidine-HCl), refolding of the totally denatured protein is required. At present, determining the best process is a trial-and-error procedure. Typical steps are careful removal of the solubilizing agent and the addition of compounds (e.g. thiols) to control the formation of disulfide bonds. Substances like polyethylene glycol have also been reported to improve folding [94, 95]. The in vitro usage of chaperone proteins has been suggested and is an area of active investigation [96, 97]. In general, however, renaturation is still problematic. Although high yields of renaturated proteins have been reported [98], recovery of activity has been low in some cases, especially for larger proteins. In addition, the kinetics of the refolding process are often slow.

An interesting approach to improve the yield and kinetics of renaturation is suggested by the work of Orsini et al. [95], in which removal of a portion of the prourokinase sequence fortuitously resulted in the doubling of the refolding rate and active product yield. If problematic regions (e.g. cysteine-rich) can be altered without affecting the desired activity, similar process improvements might be possible for other proteins.

3.2.2 Factors Influencing Formation

Although the mechanism of inclusion body formation is not well understood, a number of factors influencing their production have been identified. These include aspects of the protein molecule, the rate of protein synthesis, the cultivation conditions, and the host strain used.

Researchers have sought to find a correlation between the formation of inclusion bodies and various protein characteristics. There does not appear to be a connection between the molecular mass, number of disulfide bonds, or hydrophobicity of the protein product [99]. However, an interesting trend has been observed for proteins consisting of subunits; when all necessary subunits are produced concurrently in a cell, they are typically present in the soluble form. On the other hand, inclusion bodies result when individual subunits are produced separately [99].

The rate of protein expression appears to have a major role in determining whether or not soluble protein is produced. High rates of expression generally yield inclusion bodies [100]. Thus, the use of inducible promoters or runaway replication vectors should be expected to lead to the production of inclusion bodies.

Cultivation conditions also affect the form in which foreign proteins appear. Temperature has a significant influence; higher temperatures result in increased inclusion body formation [101]. This could be not only due to an increased rate of synthesis, but also to the relationships between temperature, the protein folding kinetics, and the growth rate of the cells. Similarly, media compositions and pH values that reduce the growth rate have been observed to lead to lower levels of inclusion body formation [93]. In some cases, special medium compo-

nents such as metal cofactors or nonmetabolizable sugars may exert direct influences on protein folding [102, 103].

Finally, the choice of host *E. coli* strain has been shown to have an impact on the formation of soluble vs particulate protein [104]. As with other aspects of recombinant cultivations that vary among strains, the basis for this influence is not known.

4 Parameters Relating to Downstream Processing

4.1 Cell Harvest and Cell Disruption

If the product is not secreted into the medium or at least exported into the periplasmic space, the cells must be harvested and disrupted to release the product. This is the case for both inclusion bodies and soluble intracellular proteins.

4.1.1 Cell Harvest

The harvesting of cells following a cultivation is basically a liquid-solid separation. Although this is a very common step in the biotechnology industry, it is by no means a simple operation on a large scale. The most frequently used methods are centrifugation and filtration [105]. For large-scale use, only continuous centrifuges such as the tubular bowl or disc models are practical. Common large-scale filtration methods include rotary vacuum drum filters, plate filter presses, and tangential and microfiltration.

It may be possible to employ genetic engineering approaches to improve this separation. One example is a mutation in the *pil* operon that results in the overproduction of pili and subsequent cell flocculation [38]. This leads to an increase in the sedimentation rate and should translate into more rapid centrifugation as well. A similar method that has been developed for yeast involves the cloning of a cell surface protein responsible for flocculation [106].

4.1.2 Cell Disruption

Both mechanical and non-mechanical methods are used to disrupt cells in large-scale processing [105, 107]. Mechanical methods such as high pressure homogenization and bead mills often result in product losses through inefficient disruption or thermal denaturation of proteins. Non-mechanical methods include those based on physical processes (e.g. sonication) and chemical effects (e.g. organic solvents and enzymatic lysis). Each of these have disadvantages; for

example, large-scale use of organic solvents has an associated explosion risk [108] and lytic enzymes are expensive [109].

Due to the importance of cell disruption for recovery of recombinant proteins, several genetic approaches have been proposed recently to improve the process with respect to efficiency, active protein yield, waste chemicals, and cost.

One such possibility is the creation of mutant host strains that have a more permeable outer membrane. For example, the amount of porin in the outer membrane of a strain of *E. coli* K12 was altered to increase the permeability [110]. This led to an increase in the export of substances out of the cell. However, mutations such as these frequently result in unhealthy cells that are unable to grow well.

A more elegant technique is to increase the permeability or disrupt the cells at an appropriate time in the cultivation (analogous to the concept of induced transcription). An example of this involves the *kil* gene of ColE1, the product of which leads to total cell lysis [111]. This gene has been placed under the control of the *lac* promoter and incorporated into a plasmid. Thus, lysis can be induced with IPTG after overexpression of the recombinant protein [112]. A similar system has been developed using the lysis gene E from bacteriophage ϕX174, under control of the λP_L promoter [113]. When this system is used together with the temperature sensitive repressor cI857, cell lysis can be induced via a temperature shift to 42 °C.

4.2 Protein Transport

In many cases, it would be desirable to secrete the recombinant product into the medium. Purification would be simpler than for an intracellular protein since the product would not be contaminated with cytoplasmic components. (It would be necessary to handle larger volumes, but this problem has been lessened by newer chromatographic methods.) In addition, the formation of inclusion bodies could be avoided and the toxic effects of some protein products on the host cell could also be reduced. Protein secretion would also reduce proteolysis, unless exoproteases are produced by the transport system.

Excretion into the periplasmic space of *E. coli* also provides many of these benefits. Proteins can be freed from the periplasmic space with gentle treatments that remove the cell wall and outer membrane.

A significant disadvantage facing protein secretion is that protein folding may be incorrect or may not occur. As discussed in the following section, this is a problem in the production of all recombinant proteins. The investigation of special reactors for promoting folding is underway in several laboratories.

Two important factors influence the transport of proteins: the type of cellular transport system and the nature of the signal (or leader) sequences that allow a protein to use that transport system.

4.2.1 Signal Sequences

Signal sequences are short peptides that allow the protein to be transported. In bacteria, these signal sequences are typically 15 to 30 amino acids long [114, 115] and are located at the N-terminus of proteins, although some, like that of hemolysin, are positioned at the C-terminus. Signal sequences form positively charged heads that are able to pass through the membrane. Between the signal peptide and the preprotein, a cleavage site for specific signal peptidases is located to allow formation of the mature protein after transport.

In addition to the leader sequences, transported proteins often have other sequences that are important for secretion. One type, located inside the protein sequence, aids in the formation of a transport competent structure. The function of other sequences, found in membrane proteins, is to stop transport. Thus, the fusion of a gene to a signal sequence does not guarantee that the product will be transported [116].

Some signal sequences that have been used to transport recombinant proteins are shown in Table 2. Well known signal sequences like *bla* (β-lactamase), *malE* (maltose binding protein), *ompA* (outer membrane protein), and *phoA* (alkaline phosphatase) come from proteins that are exported into the periplasm. Fusion proteins formed with one of these are therefore mainly transported into the periplasm, but some secretion has also been observed. Of special interest are the signal sequences *spA* (*Staphylococcus* protein A) and *malE*, since both can be used as purification tags in affinity chromatography (see Sect. 4.4).

4.2.2 Secretion Systems

Different organisms can be used as a secretion system. Bacterial hosts include *Bacillus subtilis*, *Staphylococcus aureus*, *Streptomyces lividans*, and, increasingly, *E. coli*. Important eukaryotic secretion systems are yeasts and cell cultures.

The transport of recombinant proteins by one of these hosts can be accompanied by several difficulties [114, 115]. For example, the secretion system may not be able to transport proteins that are too large or have an unfavorable distribution of charges, the wrong hydrophobicity, or similar structural burdens. Other potential problems include the saturation of export sites, competition for the signal peptidases, or lack of the proteins that support secretion.

Genetic engineering can be used to overcome some of the disadvantages of the host. The secretion machinery can be optimized or the permeability of membranes and cell walls can be changed. For *E. coli*, a series of different proteins involved in export to the periplasm are known. Most of these belong to the *sec* product family, and can be coexpressed with the recombinant protein to produce an increase in secretion. Alternatively, the membrane can be changed to

Table 2. Signal sequences and target proteins for transport in *E. coli*; the final protein location is shown as periplasmic space (P) or medium (M)

Signal sequence	Target protein	Location	Ref.
amy	amylase (*B. stearothermophilus*)	M	116
bla	proinsulin (human)	?	117
	IgG (mouse)	?	118
	β-lactamase	M	119
	epidermal growth factor (rat)	P/M	120
	triosephosphatase (chicken)	M	121
malE	gene 5 protein (phage M13)	P	122
	Klenow polymerase	P	123
	nuclease A (*S. aureus*)	P	123
ompA	colony stimulation factor (human)	?	124
	superoxide dismutase (human)	P	125
	α2 interferon	?	126
	antiviral proteins (*Mirabilis*)	M	127
	α-sarcin	P	128
	prokallikrein (human)	P	129
	nuclease A (*S. aureus*)	P	125
ompF	β-endorphin	M	130
phoA	trypsin inhibitor (bovine)	?	131
	epidermal growth factor (human)	?	132
	fusion: β-galactosidase-alk. phosphatase	M/P	133
	α-neo-endorphin	P	134
	fusion: maltose binding protein-β-galactosidase	P	135
	ribonuclease T1	P	136
phoS	growth hormone release factor (human)	P	137
spA	parathyroid hormon (human)	M	138
	insulin-like growth factor (human)	M	139
Ovalbumin	ovalbumin	?	140
Pullulanase	β-lactamase	M	141
Preproinsulin (rat)	proinsulin (rat)	?	142
Enterotoxin LTA	epidermal growth factor (human)	?	143
Synthetic	α2 interferon	P	144
Metalloprotease and helper protein	metalloprotease (with helper protein)	M	145
BRP as helper	insulin-like growth factor (human)	M	131
	cloacin	M	146
hly	hemolysin	M	147

Other abbreviations: amy = amylase, bla = β-lactamase, BRP = bacteriocine release protein, hly = hemolysin, M = medium, malE = maltosebinding protein, ompA = outer membrane protein A, ompF = outer membrane protein F, P = periplasmic space, phoA = alk. phosphatase, phoS = phosphate binding protein, spA = *Staphylococcus aureus* protein A.

yield leaky mutants. Using this approach, alkaline phosphatase [148], β-lactamase [149], and rat proinsulin [150] were secreted into the medium from *E. coli*. The major disadvantage to this method is that leaky mutant hosts are very sensitive to their environment and are difficult to grow. An interesting option is the use of bacteriocin release protein. When expression of this protein was induced, human growth hormone, which had accumulated in the periplasm of *E. coli* due to its fusion to a signal sequence, was secreted into the medium [131].

The export of proteins into the periplasmic space is of increasing interest. The advantages of the periplasmic space, which accounts for 20 to 40% of the total cell volume, include protection against cytoplasmic proteases (Sect. 3.1.4), reduced frequency of inclusion body formation (Sect. 3.2), and the proper environment for correct protein folding (Sect. 4.3). The use of the periplasmic space in industrial scale processes depends on the development of methods to open the cell wall and outer membrane without releasing cytoplasmic substances. At present, the outer membrane is often made more permeable by the addition of chemicals or osmotic shock. Reports of secretion systems in which the outer membrane leakiness is increased by plasmid-encoded products have also been published [151]. Enhanced membrane permeability may also occur fortuitously as a result of high-level expression of a foreign protein.

4.3 Protein Folding

The correct folding of a protein is essential for its biological activity. Although issues of recombinant protein structure and overproduction receive a great amount of attention, relatively little work has been done on protein folding, despite its immense commercial impact and status as an important fundamental question [152, 153]. The factors influencing protein folding are diverse and must be considered when the production of active proteins is desired [154, 155, 156].

The production of recombinant proteins leads to at least two additional problems with regard to protein folding: incorrect folding and the need to renature proteins produced as inclusion bodies.

Some proteins, especially those from eukaryotic sources, are not folded correctly in the oxidative milieu of the bacterial cytoplasm. One way to address this problem is to export the protein into the periplasmic space of *E. coli* (via fusion to a signal sequence). The periplasmic space is a reducing environment and supports correct folding of eukaryotic recombinant proteins.

The need to refold proteins from inclusion bodies can be obviated by preventing their formation. As discussed earlier (Sect. 3.2.2), this can be achieved by using low-expression systems, by changing the cultivation conditions, or by fusion to a signal peptide, among other methods.

4.4 Protein Separation and Purification

A major portion of the production costs in recombinant protein cultivations is due to separation and purification needs. Thus, improvement of these steps via genetic engineering approaches is becoming more common. Purification can be simplified by the secretion of proteins into the medium or excretion into the periplasmic space (Sect. 4.2), but even if this can be achieved, it is still necessary to isolate and purify proteins from a relatively complex mixture.

A good technique for improving protein purification is the use of tags fused to the protein product. The tag (e.g. *Staphylococcus* protein A) is chosen to allow the use of efficient separation techniques such as affinity chromatography. In order to facilitate removal of the tag following purification, a specific cleavage site can be placed between the tag and the desired protein. In Table 3, a number of different tags and their chromatographic ligands are presented.

An example of the power of this method is presented in Fig. 2. In this case, *Staphylococcus* protein A (SpA) was used as a fusion tag for the production of

Table 3. Examples of purification methods using tagged fusion proteins

Interaction	Tag	Ligand	Target protein	Ref.
Immunoaffinity	β-galacto-sidase	anti-β-gal. ab	proline carrier protein	157
Pseudoimmuno-affinity	spA	IgG	EcoRI	158
Substrate affinity	β-galacto-sidase	APTG	DNA binding protein	159
Common binding affinity	malE	starch	gene 5 protein phage M13	160
Metal chelate affinity	his_6	Ni(II)-NTA	DHFR	161
Charge	arg_5	cation exchanger	β-urogastrone	162
Hydrophobic interaction	phe_{11}	phenyl groups	β-galacto-sidase	163
Cysteine thiol interaction	cys_4	thiol groups	galactokinase	163

Abbreviations: ab = antibody, APTG = p-aminophenyl-β-D-thiolgalactoside, DHFR = dihydrofolic acid reductase, IgG = immunoglobulin G, malE = maltose binding protein, NTA = nitrilotriacetic acid, spA = *Staphylococcus aureus* protein A.

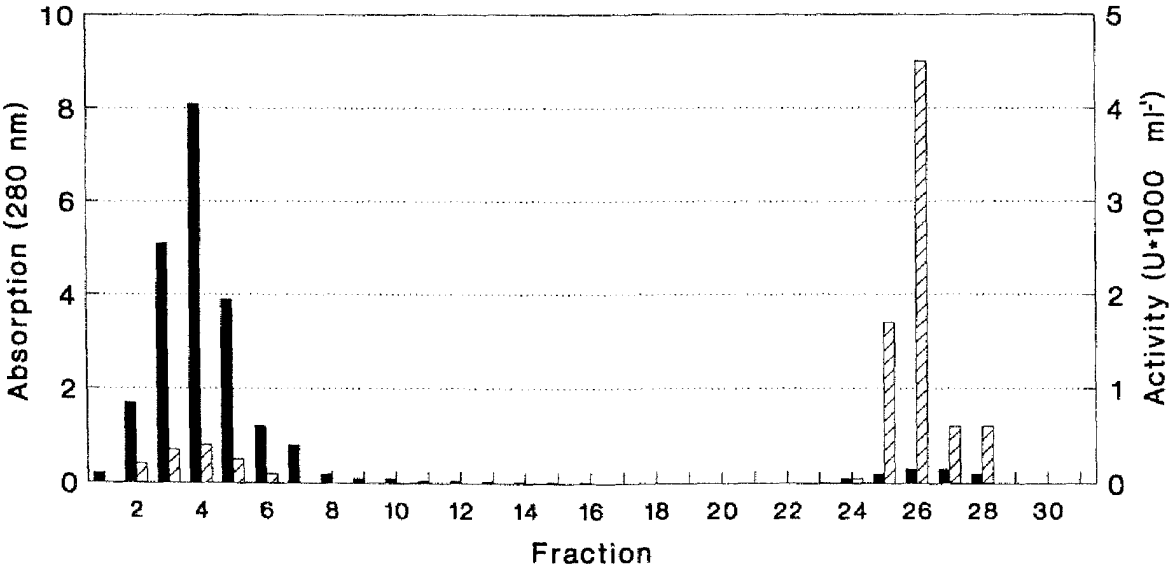

Fig. 2. Affinity chromatography of EcoRI-SpA fusion on IgG ■ Total protein (Abs.) ▨ EcoRI activity

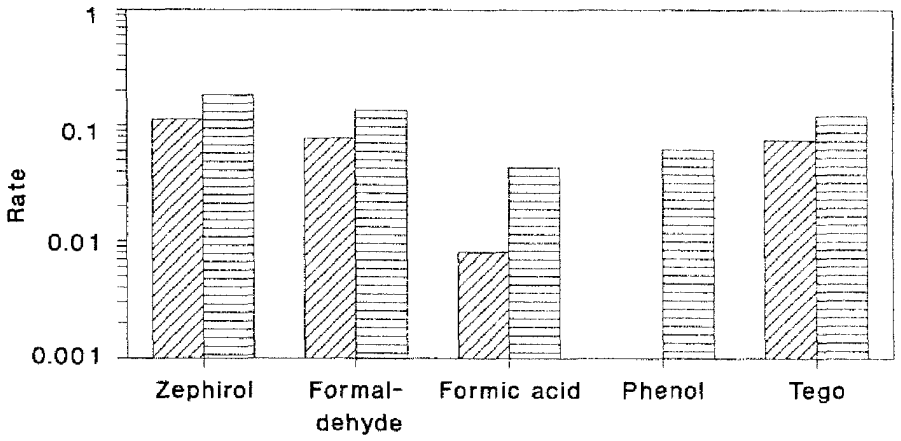

Fig. 3. Inactivation of plasmids by use of disinfectants. Rate of transformation relative to untreated samples. Concentrations: ▨ 1% ▤ 0.1%

the restriction enzyme EcoR1 [158]. The fusion protein could be enriched and purified in one step using affinity chromatography with IgG as the ligand.

4.5 Inactivation of Biological Waste

For safety reasons and for public acceptance, it is necessary to inactivate the biological waste from the production of recombinant proteins. While this is also done for many other cultivations, inactivation of recombinant microorganisms involves not only killing whole cells but also significantly reducing the level of recombinant DNA (such as transformable plasmid DNA).

A series of experiments was performed to determine the efficiency of plasmid inactivation by different methods. The intact biological activity of the plasmids was measured by comparing the transformation rate of isolated plasmid DNA before and after treating the cells. The results of these experiments (shown in Fig. 3) demonstrate that several commonly used compounds, including formaldehyde, formic acid, phenol, Tego, and Zephirol cannot inactivate plasmids when used in normal concentrations [164].

The only truly efficient method to inactivate plasmid DNA with respect to transformation is thermal incubation. This can be accomplished continuously, just as sterilization is.

5 Conclusion

In this review, we have presented a wide variety of factors and techniques that influence the productivity of recombinant *E. coli* cultivations. These parameters

exert their effects on many levels, from increased plasmid stability to facilitated product purification.

This information could be used to improve productivity by guiding the design of a vector, the choice of a host strain, or the selection of cultivation conditions and bioreactor type. In addition, the examples provided here suggest additional strategies by which the production of recombinant proteins could be enhanced. The rapid pace of research in this direction should result in many exciting developments in the next few years.

6 References

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