A new approach to increase yields and improve functionality of recombinant proteins

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Introduction and background

The ability to express and extract fully functional proteins in sufficient quantities from bacterial cultures is a prerequisite for many projects in which recombinant proteins are required for structural studies, functional characterization, as assay components or for other applications. However, low yield, poor solubility and lack of functionality are often associated with, what should be, a routine procedure.

Conventional protein production in bacterial cultures

Most frequently, bacteria are grown in shake flasks or plates using commercially-available or 'in-house' adaptations of LB, TB or M9. As illustrated in Figure 1, when supplied with a carbon source such as glucose, bacteria grow and divide rapidly at their maximum growth rate. However, as uncontrolled growth and fast aerobic metabolism deplete oxygen and glucose levels and alter the surrounding pH, bacteria soon switch over to anaerobic metabolism.
Uncontrolled nutrient supply, accumulation of harmful metabolites, oxygen depletion and poor pH control limit protein yields, while the time window for expression induction can be very short, requiring frequent O.D. measurements to determine the optimum moment.
In addition, the quality of proteins expressed and modified under sub-optimal conditions is compromised. For example, folding and other post-translational machinery, such as chaperones or phosphorylases, may be unable to match the fast rate of synthesis. Insufficient levels of these accessory proteins, together with oxygen depletion, increase the risk of poor solubility or low activity of the end product.

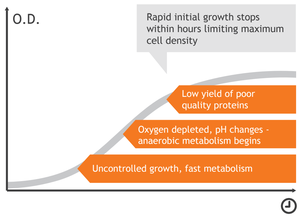


Fig. 1. Typical bacterial growth curve in conventional growth media. Narrow time windows for induction and proteins often expressed under sub-optimal metabolic conditions result in low yields associated with poor solubility and/or functionality.

Rethinking the approach to cell culture

Figure 2 suggests that, if bacteria are supplied with the key elements to support growth, control pH and regulate glucose availability, cells will grow steadily to reach much higher densities. In relation to protein expression this enables induction at a high cell density and ensures that cells continue in linear growth for many hours after induction.- leading to significantly higher yields of protein
Since proteins are expressed and modified under optimal metabolic conditions (slower synthesis rate, improved protein folding) these higher yields should be associated with improved solubility and/or functionality.

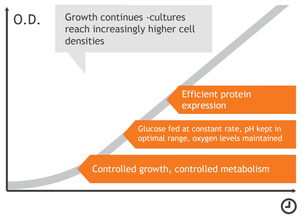


Fig. 2. A theoretical growth curve assuming that bacteria are supplied with the key elements to support growth, control pH and regulate glucose availability. Cells remain in linear growth, proteins are expressed under optimal metabolic conditions resulting in high yields and improved functionality.

Technology principle

EnPresso® growth systems offering a novel approach to control the feeding, and thereby growth, of microbial cultures in order to achieve high density cell cultures, predictable induction times and hence higher yields of functional proteins. After dissolution in sterile water and addition of the inoculum, a glucose-releasing agent breaks down long chains of polysaccharides which were contained within each tablet, releasing glucose units at an optimal rate to feed the microbes. Controlling glucose release (the 'feed') controls the rate of growth enabling microbes to remain in linear growth. Each tablet also contains key elements to support growth and maintain pH thereby ensuring optimal metabolic conditions for protein expression.

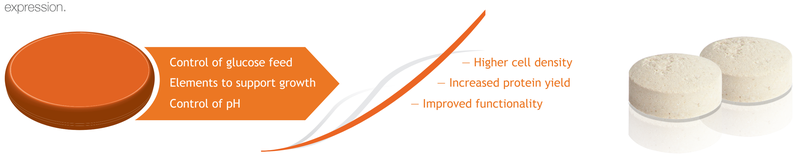


Fig. 3. EnPresso growth systems are supplied as pre-sterilized tablets ready to dissolve in sterile water. Each tablet contains polysaccharides, growth elements and buffering components to support optimal growth and metabolism. Controlled release of glucose from the polysaccharides maintains cells in linear growth.

Methods

Recombinant proteins – from pre-culture to harvest within two days

Yields of recombinant proteins from *E. coli* cultures grown in conventional growth media were compared with yields from the identical expression system and *E. coli* strain grown in a commercially-available EnPresso growth system (BioSilta Oy). Yields from EnPresso B were compared against yields from LB media. Yields from EnPresso B Defined Nitrogen-free (a chemically-defined growth system specifically designed for preparation of 15N-labelled proteins) were compared against yields from a variety of nitrogen-free growth media.
Established protocols were followed for growth, induction and harvesting from conventional growth media. Protocols supplied by the manufacturer were followed for growth, induction and harvesting from each EnPresso growth system. A typical protocol is shown in Figure 4.

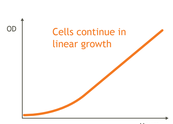


Fig. 4. Pre-sterilized EnPresso growth system tablets are dissolved into sterile water - no media autoclaving is required. The optimized protocol reduces hands-on time – no O.D. monitoring is required. In a typical workflow, cultures are grown overnight, induced the following morning and harvested next day.

Plasmid production – product development and optimization

Low yields and poor quality are typical challenges associated with the production of plasmids. Data presented in this poster shows the potential to apply a similar approach to increase yields and improve quality as that used for production of recombinant proteins. Preliminary results obtained during product development and protocol optimization to produce low and high copy number plasmids in two of the most commonly-used *E. coli* strains are presented.

Results: increased protein yields and improved functionality

Enzymes expressed in greater amounts with higher specific activity

Figure 5a shows the substantial increases in yield of several haloalkane dehalogenase variants when *E. coli* cultures were grown in an EnPresso B growth system. By controlling glucose availability, the bacteria were kept in linear growth, making it possible to reach cell densities 15-30-fold higher than those achievable in LB media before induction of protein expression. Figure 5b demonstrates the improvement in protein functionality, in this case measured in terms of specific activity, since proteins are expressed under optimal metabolic conditions. During this work it was demonstrated that the expression profile remained constant during the 24 hour induction period (result not shown), a contributory factor leading to the significant improvement in yield of active enzyme per culture volume. Table 1 shows further examples of substantial increases in protein yield from cultures grown in EnPresso B.

>30-fold increase in active enzyme

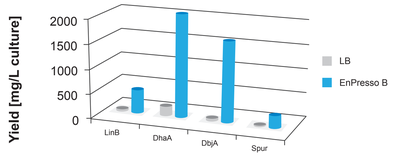


Fig. 5a. Yields of haloalkane dehalogenases expressed in E. coli grown in an EnPresso B growth system compared to yields from the same expression system grown in commercially-available LB media.

Results courtesy of Lukáš Chrást, Masaryk University, Czech Republic

>3.3-fold increase in specific activity

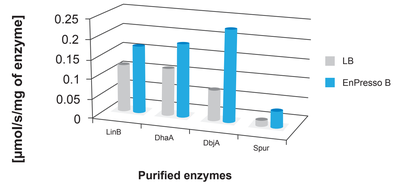


Fig. 5b. Specific activities of purified haloalkane dehydrogenases expressed in E. coli and grown in an EnPresso B growth system compared to the specific activities using the same expression system and purification method, but grown in commercially-available LB media. Specific activity determined with 1,2-dibromoethane (LinB, DhaA and DbjA) and 1-bromobutane (Spur).

Yield increase compared to standard LB medium	Protein	Results courtesy of:
66 -fold (mg/mL)	Human 15-1 lipoxygenase (toxic)	T. Horn, Charité, Berlin, Germany
20 -fold (mg/mL)	Human cyclophilin-A	M. Wear, Edinburgh Protein Production Facility, UK
>15 -fold (mg/mL)	Fusion peptide 1	M. Crampton, CSIR, Pretoria, South Africa

Table 1. Data from independent labs producing recombinant proteins from E. coli cultures grown in EnPresso B. Note the ability to significantly increase yield of a lipoxygenase – previously expressed in very low amounts due to the toxicity of the protein.

^{15}N-labelled protein produced in greater quantities for NMR analysis

Poor growth, low levels of expression and insufficient labelled protein are the most frequent challenges faced when using minimal medium to prepare proteins for structural characterization by NMR analysis. Growing *E. coli* in EnPresso B Defined Nitrogen-free, a chemically-defined growth system ensures that cultures can reach significantly higher densities before induction (Figure 6a). This is a particular advantage when expressing proteins that may be toxic to *E. coli*. Figure 6b shows how an EnPresso growth system yielded a cytokine fragment that had been impossible to produce using LB or M9 growth media. Table 2 shows further examples of labelled proteins prepared for NMR analysis in greater quantities than had been achievable with other defined growth media.

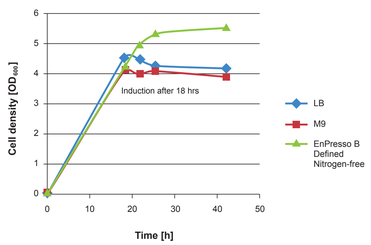


Fig. 6a. Cellular growth of cultures with toxic target protein (cytokine fragment) using different media – higher cell density with EnPresso B Defined Nitrogen-free

Results courtesy of Professor Birthe Kragelund, Dept. of Biology, University of Copenhagen, Denmark

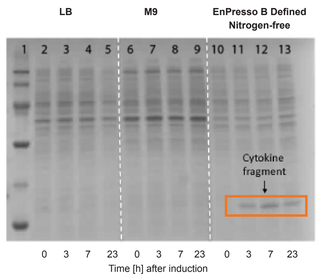


Fig. 6b. SDS-gel: samples of soluble protein produced with LB, M9 and EnPresso B Defined Nitrogen-free after different times of induction

Yield increase compared to standard growth media (mg/mL)	Protein	Results courtesy of:
64-fold	Protein XX	B. Davis, Vernalis, UK
4 to 5-fold	Three target proteins	Major pharmaceutical company, Italy
5-fold	SPSB2 as GST fusion	E. Leung, Monash Institute of Pharmaceutical Sciences, Australia
5-fold cf. complex medium 7-fold cf. complex medium	Protein X Protein Y	Major pharmaceutical company, UK
>3-fold cf. modified M9 >2-fold cf. modified M9	PDZ domains as fusions	A. Diehl, Leibniz Institute for Molecular Pharmacology, Germany

Table 2. Data from independent labs producing ^{15}N-labelled proteins for NMR analysis from E. coli cultures grown in EnPresso B Defined Nitrogen-free

Preliminary results: increased yields of high quality plasmids

Low yields and poor quality are typical challenges associated with the production of plasmids in shake flask cultures. Work is therefore ongoing in the BioSilta laboratories to investigate the potential of optimizing an EnPresso growth system to increase yields of high quality plasmids from *E. coli* cultures. Preliminary results obtained during protocol development to produce low and high copy number plasmids in two of the most commonly-used *E. coli* strains are presented below.

Increased yields of high and low copy number plasmids from *E. coli* shake flask cultures

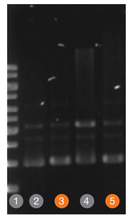
50 mL culture in 500 mL Erlenmeyer flask	LB media		Prototype EnPresso 'plasmid growth system'		
	mg/L	mg/L/OD	mg/L	mg/L/OD	Fold increase
DH5alpha/pUC19	3.6	1.8	41.8	3.34	11.6
DH10B/pUC19	5.8	1.45	32.6	2.17	5.6
DH5alpha/pETM-22	2.9	0.58	11.5	0.69	4.0
DH10B/pETM-22	2.9	0.73	12.5	0.75	4.3

- Minimum 4-fold increase of low copy number plasmid
- Up to 12-fold increase of high copy number plasmid
- pDNA per 50 mL culture 500-2000 µg
- Results scalable to larger culture volumes

Table. 3. When compared with E. coli cultures grown in LB media, cultures grown in a prototype EnPresso 'plasmid' growth system' provide significantly higher yields of high copy number (pUC19) and low copy number (pETM-22) plasmids. Controlled growth rate within the EnPresso culture enables cells to reach higher densities before harvesting. There are also indications that the copy number per cell is increased.

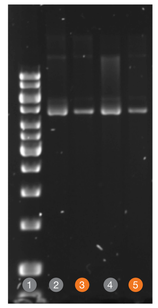
Improved quality compared to plasmids from cultures grown in LB media

puc19



1. Markers
2. LB - DH5alpha
3. EnPresso 'plasmid' - DH5alpha
4. LB – DH10B
5. EnPresso 'plasmid' - DH10B

pETM-22



1. Markers
2. LB - DH5alpha
3. EnPresso 'plasmid' - DH5alpha
4. LB – DH10B
5. EnPresso 'plasmid'- DH10B

Fig. 7. When compared with E. coli grown in LB media, cultures grown in a prototype EnPresso 'plasmid growth system' produce both high and low copy number plasmids with a higher percentage of supercoiled (covalently closed circular) plasmid implying lower levels of nicked pDNA. The controlled growth rate within the EnPresso culture enables cells to reach a higher density before harvesting and plasmids are produced under optimal metabolic conditions – avoiding oxygen depletion and significant changes in pH.

Protocols for small-scale production or screening applications

6 hour cultivation	0.35 mL cultures in closed Eppendorf tubes using a Thermomixer		0.3 mL cultures in closed Eppendorf tubes using a Vortex Genie 2	
	µg DNA	ng/µL	µg DNA	ng/µL
DH5alpha/pUC 19	3.4	68	3.6	72
DH5alpha/pET15	1.1	22	1.4	28
DH10B/pUC19	2.9	58	2.9	58
DH10B/pET15	1.8	36	1.4	28

Table. 4. Data in the table above shows that high and low copy number plasmids can be obtained in good yields from EnPresso 'plasmid growth system' cultures using controlled shaking conditions to provide adequate aeration in the closed Eppendorf tubes. Work is therefore continuing to develop reliable protocols suitable for plasmid production in these smaller culture volumes and using alternative formats such as microplates or deepwell plates as typically required for high throughput screening experiments.

Conclusions

- Utilizing enzyme-controlled glucose release to control the growth of *E. coli* cultures expressing recombinant proteins significantly increases protein yield and improves parameters such as the functionality, activity and solubility of the final recombinant product. Protein yields have been increased significantly when compared to yields from cultures grown in conventional LB or M9 media.
- Independent laboratories are demonstrating that, by optimizing bacterial growth conditions using an EnPresso growth system, even proteins that would normally be toxic to the cultures can be expressed successfully.
- Scientists using EnPresso B Defined Nitrogen-free are easily producing sufficient quantities of 15N-labelled proteins from single cultures prior to NMR analysis.
- Preliminary data applying a similar approach to increase yields of high and low copy number plasmids from *E. coli* cultures suggest that utilization of an EnPresso growth system optimized for plasmid production could not only enable cultures to reach higher cell densities but may also increase the plasmid number per cell. Both factors would contribute to higher yields, providing a more cost-effective, time-efficient solution for routine plasmid production.

Acknowledgments

BioSilta would like to express their appreciation to all scientists who have contributed during the development and testing of EnPresso growth systems and who have allowed their results to be presented in this poster.