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Bioprocessing of Therapeutic Proteins from the Inclusion Bodies of *Escherichia coli*

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Escherichia coli has been most extensively used for the large-scale production of therapeutic proteins, which do not require complex glycosylation for bioactivity. In recent years tremendous progress has been made on the molecular biology, fermentation process development and protein refolding from inclusion bodies for efficient production of therapeutic proteins using *E. coli*. High cell density fermentation and high throughput purification of the recombinant protein from inclusion bodies of *E. coli* are the two major bottle necks for the cost effective production of therapeutic proteins. The aim of this review is to summarize the developments both in high cell density, high productive fermentation and inclusion body protein refolding processes using *E. coli* as an expression system. The first section deals with the problems of high cell density fermentation with an aim to high volumetric productivity of recombinant protein. Process engineering parameters during the expression of ovine growth hormone as inclusion body in *E. coli* were analyzed. Ovine growth hormone yield was improved from 60 mg L^{-1} to 3.2 g L^{-1} using fed-batch culture. Similar high volumetric yields were also achieved for human growth hormone and for recombinant bonnet monkey zona pellucida glycoprotein expressed as inclusion bodies in *E. coli*. The second section deals with purification and refolding of recombinant proteins from the inclusion bodies of *E. coli*. The nature of inclusion body protein, its characterization and isolation from *E. coli* has been discussed in detail. Different solubilization and refolding methods, which have been used to recover bioactive protein from inclusion bodies of *E. coli* have also been discussed. A novel inclusion body protein solubilization method, while retaining the existing native-like secondary structure of the protein and its subsequent refolding in to bioactive form, has been discussed. This inclusion body solubilization and refolding method has been applied to recover bioactive recombinant ovine growth hormone, recombinant human growth hormone and bonnet monkey zona pellucida glycoprotein from the inclusion bodies of *E. coli*.

Keywords. Fed-batch fermentation, Volumetric productivity, Recombinant protein, Inclusion body, Refolding, Purification

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Symbols and Abbreviations

ATP	adenosine triphosphate
ATR-FTIR	attenuated total reflectance Fourier transform infrared
bGH	bovine growth hormone
BFGF	basic fibroblast growth factor
bmZPC	bonnet monkey zona pellucida glycoprotein C
βgal	beta-galactosidase
CD	circular dichroism
CDNA	complementary deoxyribonucleic acid
CER	carbon dioxide evolution rate
C/N	carbon to nitrogen ratio
DEAE	diethylaminoethyl
DO	dissolved oxygen
DTT	dithiotheritol
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
GSH	glutathione reduced
GSSH	glutathione oxidized
hGH	human growth hormone
IFN-γ	interferon-γ
IGF-1	insulin like growth factor-1
IL-1β	interleukin-1β
IL-2	interleukin-2
IPTG	isopropyl thio β-D galactopyranoside
kDa	kilodalton
LB	luria bertani
M	mass
$NADH_2$	nicotinamide adenine dinucleotide dihydrogen
Ni-NTA	nickel-nitrilotriacetic acid
OD	optical density at 600 nm
oGH	ovine growth hormone
PAGE	polyacrylamide gel electrophoresis
PDI	protein disulfide isomerase
PEG	polyethylene glycol
PHB	polyhydroxybutyrate
PMSF	phenylmethylsulfonyl fluoride
PO_2	oxygen partial pressure
PPI	prolyl-peptidyl isomerase
r-bmZPC	recombinant bonnet monkey zona pellucida C
rhaBAD	rahmnose BAD
r-hGH	recombinant human growth hormone

RIA	radioimmunoassay
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
r-oGH	recombinant ovine growth hormone
SDS	sodium dodecyl sulfate
S-200 HR	Sephacryl 200 high resolution
TCA	tricarboxylic acid
tRNA	transfer ribonucleic acid
V	volume
ZP	zona pellucida
ZPA	zona pellucida A
ZPB	zona pellucida B
ZPC	zona pellucida C
μ	specific growth rate

1 Introduction

The ultimate goal of recombinant fermentation research is the cost effective production of desired protein by maximizing the volumetric productivity, i.e., to obtain the highest amount of protein in a given volume in the least amount of time. Such bioprocessing for recombinant protein using genetically modified organisms requires a stable high-yielding recombinant culture, a high productive fermentation process and cost effective recovery and purification procedures. *Escherichia coli* species have been most widely used as host for the expression of recombinant proteins [1, 2]. Advantages of using *E. coli* as expression system is the enormous amount of data available on its cell biology, fermentation process development and its ability to produce large quantities of recombinant proteins in an inexpensive way. The successful large-scale cost-effective production of insulin by Eli Lilly (USA) and bovine growth hormone by Monsanto Corporation (USA) attest to the versatility and economic potential of *E. coli*-based therapeutic protein production. Although *E. coli* cannot be used to produce complex glycoproteins or proteins having multiple disulfide bonds, in past 20 years recombinant DNA technologies have enabled us to produces huge quantities of therapeutic proteins that might otherwise have been difficult [3, 4].

Recombinant protein expression using *E. coli* as host is frequently associated with the formation of intracellular aggregates as an inclusion body [5]. The volumetric yield of the protein is thus is a function of both unit cell concentration and specific cellular protein yield. Optimization of high cell density fed-batch fermentation processes is thus one of the key steps for enhancing the volumetric yield of recombinant proteins [6, 7]. High level expression of protein in the form of an inclusion body facilitates the isolation of the protein of interest from the cytoplasm at the cost of its native structure. Renaturation of recombinant proteins from inclusion bodies into the bioactive form is cumbersome, results in low recovery of the final product and also accounts for the major cost in over-

all production of recombinant proteins [5, 8]. However, in the cases where a simple high-yielding protein refolding process is developed for the aggregated recombinant protein, high-level expression of protein as inclusion body provides a straightforward strategy for the cost-effective production of therapeutic protein. Thus, in spite of the problems associated with the inclusion body in *E. coli*, they have been extensively used for the commercial production of therapeutic protein. High cell density fermentation and improved refolding of the inclusion body proteins are thus the two major bioprocess engineering considerations for enhancing the overall yield of recombinant proteins from *E. coli*.

The objective of the present review is to emphasize the importance of high productive fermentation as well as high throughput purification of bioactive therapeutic protein from the inclusion bodies of *E. coli*. Understanding of basic biological aspect of the expression system at the molecular level and translating this information at process level is imperative for efficient and cost-effective production of therapeutic compounds. Parameters that influence the high cell density fed-batch aerobic growth of *E. coli* while maintaining a stable plasmid of interest have been analyzed. Novel ways of fed-batch fermentation process considering most of these factors have been discussed in detail to maximize the volumetric yield of recombinant ovine growth hormone expressed as inclusion body in *E. coli*. Solubilization and refolding of inclusion body protein to the bioactive conformation severely limits the overall efficiency of the therapeutic protein production from *E. coli*. Solubilization of the inclusion body protein without disturbing the existing native-like secondary structure while using a low concentration of a chaotropic salt, its refolding and purification into bioactive forms have been described. Finally the novelty of high cell density fermentation processes and improved refolding of inclusion body proteins have been applied to a few other proteins expressed in *E. coli* and process development strategies have been discussed. Apart from reviewing the recent trends in bioprocessing of recombinant protein from *E. coli*, the review discusses the fermentation and inclusion body protein refolding process developed at the National Institute of Immunology, New Delhi.

2 Parameters Influencing Productivity of Therapeutic Protein from *E. coli*

Numerous genetic and environmental factors influence the expression of cloned gene product in recombinant *E. coli* which is most frequently used prokaryotic expression system for the production of heterologous proteins [9]. At the molecular level, strength of transcriptional promoters, plasmid stability, copy number, mRNA stability, translational efficiency, localization, status and the stability of the expressed foreign protein in the host influences the expression levels. These factors influence the metabolic state of the host during gene expression, which in turn, can be controlled and manipulated during fermentation to maximize the yield of the expressed protein [10]. The expressed protein, depending on its localization, can be purified and recovered in the bioactive form. It is interesting to note that even though *E. coli* does not provide an oxidizing environment for disulfide bond formation leading to the aggregation

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