A Review on: Recent Advances in Production of Recombinant Proteins Using High Cell Density Fermentation of *Escherichia coli*

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Abstract -In upcoming years, there is significant increase in the demand of recombinant proteins. As a consequence, the processes used to produce these proteins must be able to meet market requirements. This review is focused on the production of recombinant proteins by high cell density fermentation. Recombinant protein (RPP) production in *E. coli* is a keystone of modern bioprocessing, especially for biopharmaceutical production. Nearly all the approved industrial recombinant proteins (IRP) are formed in *E.coli* because of its well-known genetics, fast growth, and high yield of production. The main aim of this review is to summarize recent advances in developments of high cell density/high productive fermentation of recombinant protein using *E.coli* as an expression system. Impact of process parameters on fermentation processes are discussed, and alternative methods that solve the limitations are reviewed together with the methods that yielded in higher productivity of *E. coli* process. The factors which affect high cell density fermentation are DO, pH, O2 supply, Temperature, aeration, agitation, and composition of media, feeding, and induction strategy and how to overcome this issues are discussed in this review paper.

Key Words: *E. coli*, Recombinant proteins, High cell density culture

1. INTRODUCTION

Biopharmaceuticals are large therapeutic proteins obtained by biotechnological processes (Faustino et al., 2016). These biopharmaceuticals are large and complex protein molecules derived from mammalian cell lines, plants, microorganisms, and genetically modified cells (Valderrama-rincon et al., 2012). These are therapeutic drugs produced using a biotechnological process or by using biopharmaceutical techniques (Rodríguez et al., 2014). Biopharmaceutical produced before the 1990s were proteins with unchanged signal sequence or murine antibodies. The techniques are genetic engineering, Hybridoma technology, rDNA Technology (Sekhon, 2010).

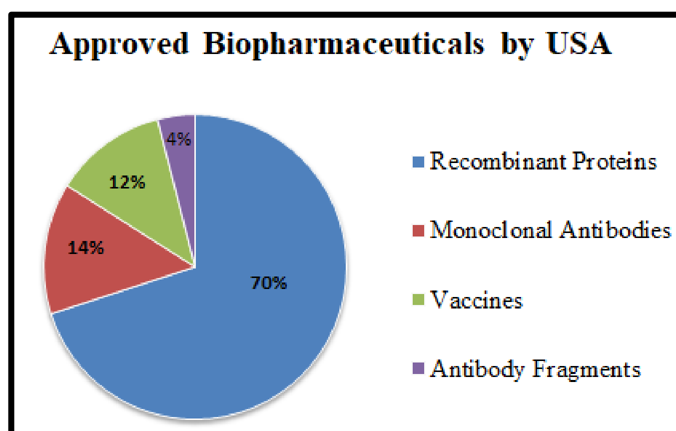

Fig 1: Graphical representation of approved biopharmaceuticals by USA and EU

But the approved biopharmaceutical products which are produced from 2004 to 2013 are produced by mammalian cells, *Escherichia coli, Saccharomyces cerevisiae*, transgenic animals, plants, and insect cells. These biopharmaceuticals are produced by genetic engineering techniques such as rDNA technology. In 2006, the USA and EU have approved 162 biopharmaceuticals and from that 70%, 14% and 12% are recombinant proteins, monoclonal antibodies, and vaccines respectively (Osasto & Sektion, 2014). The advanced biopharmaceuticals are produced using monoclonal antibodies, chemically defined cells, or by genomic manipulation (Hamrang et al., 2013). These biopharmaceuticals are used in the treatment of diabetes, myocardial infarction, congestive heart failure, cerebral apoplexy, multiple sclerosis, neutropenia, thrombocytopenia, anemia, etc. and for that human insulin, interferons, human growth hormones, and monoclonal antibodies are currently produced around the world (Sekhon, 2010). In 1982, biosynthetic Insulin was made via rDNA technology and it was the first vaccine

approved for therapeutic use. These biopharmaceuticals are produced using two processes such as the Upstream and Downstream process. The upstream process means the production of biopharmaceuticals using mammalian cell culture or by using microbial fermentation techniques(Markov, 2015).

2. What is Fermentation technology?

Upstream process or Fermentation technology is defined as the production of biopharmaceuticals or other molecules by using microbial growth. For cell growth and biopharmaceutical production many parameters such as cell line selection, media selection growth conditions are required to optimize(Faustino et al., 2016). The aim of the fermentation is the conversion of substrates into the product of interest which required optimized parameters and bioreactors (Valderrama-rincon et al., 2012). In the process of fermentation, many factors are required to consider during fermentation like process type (batch, fed-batch, continuous, etc.), pH, Temperature, and agitation, DO, and decontamination. The fermentation process involved whole living cells (microbe, mammalian, plant, yeast, etc.) for the production of commercial bio-products. The products of the fermentation process are of many types: wine, beer, alcohol, butyl alcohol, acetone, lactic acid, antibiotics, cheese, biofuels, vitamins, amino acids, solvents, and biological insecticides and pesticides(Markov, 2015). These bio-products can be either cell itself, a cell metabolite from natural or genetically improved strain or it can be a recombinant product produced using recombinant DNA technology in a living organism or cell (Pumphrey & Julien, 1976). The knowledge of microbiology, biochemistry, genetics, chemistry, chemical, and bioprocess engineering is required to develop the fermentation process. The fermentation process has been exploited over the century at industrial scale for the production of commercially valuable products broadly, for the production biomass, microbial enzymes, microbial metabolites, recombinant products, fermented milk products, numerous chemicals such as amino acids, polymers, organic acids and also many products are added into food as flavors, vitamins, colors, preservatives, and antioxidants(Kujau & Pliickthun, 1996). The fermentation process is carried out by using the following steps (Salehmin et al., 2013):

a. Organisms used in the fermentation process: For the production of enzymes or antibiotics there are different types of microorganisms such as bacteria, fungi, yeast, mammalian cells are used. The organisms should be convert substrate into product and to give a high yield, grow rapidly on less expensive media, the organism is stable to genetic manipulation, and is non-pathogenic.

b. Fermentation media: To produce the desired product microorganism's needs nutrients called the substrate. These nutrients are required for microbial growth and they are provided in the form of media. The standard media contains a carbon source, nitrogen source, minerals, growth factors, precursors, inhibitors, and trace elements.

c. Fermentation systems: The fermentation reaction takes place in a vessel called fermenters or bioreactors. These vessels are closed and available from 1Lit to 1000 Lit. The main purpose of these types of vessels is to provide controllable parameters such as pH, Temperature, Dissolved oxygen, Agitation for the growth of microbial or mammalian cells.

3. Types of Fermentation process:

Generally, the fermentation process is carried out as Batch, Fed-batch, and continuous process. The batch process involves a closed system of a culture in which the inoculum, nutrients, and medium are added initially in the bioreactor and there is no further addition or removal of media. In the batch culture, the volume of broth in a bioreactor is constant during cultivation. The Fed-Batch process is the modified version of the batch process where microorganisms are grown under the batch process for a certain amount of time and then feeding is done stepwise or continuously with the medium. High cell densities are a basis for the high volumetric yield of r-protein and are not obtained in a 'batch process. Because the batch process contains all substrates at the beginning which causes substrate inhibition and unbalanced growth and due to these cell density decreases. But when we continuously feed concentrated sugar solution or another carbon source to a culture in a bioreactor then we can achieve high cell density and this process is called the "Fed-batch process"(Pilarek, 2010). In the fed-batch culture broth volume in a bioreactor is not constant. In the continuous process, fresh nutrients are added to the vessels with simultaneous removal of an equal amount of culture

liquid. In the continuous culture volume of culture broth in the bioreactor is constant during cultivation (Salehmin et al., 2013).

4. Role of rDNA technology in industrial recombinant proteins production:

In the 20th century, the use of several protein molecules originating from an animal source for example insulin, asparaginase, glucagon, growth hormone, etc. was experienced(Faustino et al., 2016). But isolating and extracting the very small amount of protein molecules direct from the animal source was a very laborious and expensive process. This reinforced the bio-pharma industries to look for another source of protein. At that time, the expression of heterologous proteins in micro-organisms utilizing recombinant DNA technology was found as a very efficient method. The industrial recombinant proteins (IRPs) are developed by genetically modified organisms. Many groups have worked on the production of r-proteins from *E.coli* by using HCDC. There is no doubt that the demand for r-Proteins has increased in several fields. Most of these r-proteins are produced in bacteria, yeast, mammalian cell line, or even human cells. For the production of heterologous protein mostly *E.coli* used as a host of choice due to its easier culture technique, well-known genetics and it is easier to grow it to a high-density culture by using inexpensive carbon and nitrogen sources, which makes it a cost-effective and efficient way to produce a recombinant protein(Morowvat et al., 2015). The IRPs are enzymes and structural proteins that are used for the production of food, beverages, bioethanol, biodiesel, biofertilizers and biopesticides, cosmetics, and valuable chemicals. These IRPs are generated by using genetic engineering techniques. Mostly today all IRPs are produced by rDNA technology. These recombinant proteins are produced in four steps: a) Cloning of GOI into a suitable vector, b) Transformation of host cells, c) Synthesis of desired protein under controlled parameters, d) Recovery and purification of the product(Overton et al, 2014). r-DNA uses three main tools such as 1. Enzymes - restriction enzymes, polymerases, and ligases 2. Vectors and 3.Host organism (P. V. Pham, 2018). The most common types of vectors are the plasmid vector and bacteriophage(Pham et al, 2018). For the formation of rDNA products, *E.coli* is frequently used as a host system. To achieve the high intracellular concentration of the desired product there is a need to be produced a high concentration of cells in the reactor(Rabert et al., 2013).

4.1 *E.coli* as an expression system for the production of r-Proteins

Production of recombinant proteins needs a biological framework, which is known as the expression system(Ferrer-Miralles et al., 2009). The expression systems used for the production of r-proteins are both prokaryotic and eukaryotic cells such as mammalian, plant, bacterial, yeast & insect cell expression systems. *Escherichia coli* or *E.coli* is a gram-negative enterobacterium, which is mainly found in the large intestine. Yet, there are many expression systems available to date; *E.coli* is used as a bacterial expression system for the production of several r-proteins because of its low cost, rapid growth, good productivity, well-known genetics. The main drawback of *E.coli* is; it lacks a secretion system for the release of protein produced to the media. *E.coli* leads to the expression of recombinant protein in the form of inactive protein aggregates known as "Inclusion bodies" which shows incorrect folding of protein & improper formation of a disulfide bond. These protein aggregates need to be re-solubilize & refolded again, to bring the protein to its active form(Agarwal et al., 2020). It has been proved that for all foreign protein production *E.coli* may not be useful but it can be used for the production of human proteins such as Proinsulin, HGH, interferon-gamma, and antibody fragments(Agarwal et al., 2020). The first r-human insulin was formed in *E.coli* & later in 1982, approval was approved for the treatment of diabetic patients. The acceptable protein-based therapeutic drugs manufactured by *E. coli* are hormones (human insulin and insulin analogs, calcitonin, parathyroid hormone, human growth hormone, glucagon, somatotropin, and insulin growth factor 1), interferons (alfa-1, alfa-2a), alfa-2b, and gamma- 1b)(Tripathi et al., 2009). Optimum production of r-protein in *E.coli* depends on factors such as the selection of strain, plasmid stability, induction efficiency, host-vector interaction, biomass concentration, protein stability. *E.coli* cells serve as a host for vectors which are fermented appropriately for the expression of recombinant product. They also mentioned that the *E.coli* host has a switchable expression system that means they are first fermented to high cell densities and the rDNA expression is switched on(Wolf-Dieter Deckwer et al 2007). When we used *E.coli* as an expression system then we can achieve higher protein yield by using high cell density fermentation.

5. Production of r-Proteins from *E.coli* using High cell density fermentation:

There is a need to produce a high yield of IRPs and for that; the cells are grown at high densities, mostly through fed-batch fermentation. Growing the recombinant *E.coli* cells to HCDC is a method of choice because the high volumetric productivity of recombinant protein is associated with it. The advantages mentioned by Panda et al., 2003 of high cell density cultivation are as follows(Panda et al, 2003):

a. Increasing productivity, such as higher volumetric productivity.
b. Making the downstream process easier by reducing culture volume.
c. Facilitated cell separation. Improved yield in product recovery
d. Reduced amount of wastewater
e. Lower production cost
f. Reduced investment for equipment.

By using a fed-batch process, we can produce a high concentration of cells and also minimize problems such as substrate and product inhibition, dissolved oxygen limitation, etc. Various techniques have been developed for HCDC, including consideration of host cell design, adjustment of recombinant protein expression, medium composition, growth methodologies, and even process control and analysis for increase cell densities of heterologous strains in Fed-batch cultures to more than 100 gm/L (DCW)(Shiloach & Fass, 2005). The HCDC technique is used to improve productivity, reduce culture volume, to decrease manufacturing cost, reduce wastewater, etc. In the HCDC technique, maximum cell concentration was achieved which increases the volumetric productivity of IRPs(Dorka, 2007). The main objective of the HCDC technique is to achieve a high growth rate by optimizing the feeding strategy. There is a need to control the growth rate of cells because it affects the formation of byproduct, plasmid stability, and cell concentration, etc. For, attaining high- density culture, growth media should be chosen wisely. The media should meet the demands of cell growth and the production of recombinant protein. Fed-batch strategies are preferred for attaining HCDC. According to Eiteman and Altman 2006, when working with HCDC, requirements of nutrition initially added to basal media because most of the higher concentration of media components can inhibit *E. coli*. The second problem is the precipitation of the medium components, which can hamper fermentation, downstream, and the purification process. To overcome these problems, you need to design a good media strategy and a feed strategy(Eiteman & Altman, 2006). Fed-batch cultures can produce more than 50 g/L (DCW) of *E.coli*. In the HCDC technique, increasing cell concentration produces some problems such as decreasing dissolved oxygen, plasmid instability and the most important is acetate formation(Wang & Lee, 1997). At the time of HCDC *E.coli* secretes 10-30% of carbon in the form of acetate. Glucose is the generally used carbon source for *E.coli* which is metabolized directly by the glycolysis pathway. Acetate accumulation takes place when *E.coli* cells as cultivated in a higher concentration of glucose, where rapid uptake of glucose by *E.coli* effects in the formation of biomass and diverts acetyl-CoA from the TCA cycle to acetate. When there is a lack of dissolved oxygen (DO) in media, the *E.coli* cells activated fermentation pathways which in results cause accumulation of acetate. Acetate accumulation acidifies the environment due to which inhibition of cell growth takes place, resulting in low productivity of biomass as well as protein. Acetate also has a strong negative effect on the intracellular proteins of cells. Acetate also affects the pH of the environment. When pH is below 5, cell disruption takes place with protein and DNA denaturation.

6. Factors affecting the high cell density fermentation process:

The factors which affect HCDC are DO, pH, O2 supply, Temperature, aeration, agitation, and composition of media, feeding, and induction strategy which can affect the high cell density fermentation(Renge et al., 2012).

a. Temperature: Normally, *E. coli* grows at a temperature ranging from 23 °C to 40 °C. While the optimal temperature for *E. coli* growth is 37 °C. For *E. coli* HCDC, the control of temperature is much more important due to significant heat release despite limited heat transfer due to high viscosity. The growth of *E. coli* and the production of the r-protein are affected by the temperature provided to it.

b. Dissolved Oxygen: Recombinant protein production in *E.coli* takes place at aerobic conditions because at the anaerobic condition it

provides less energy for metabolic process & protein production. *E.coli* consumes oxygen from the growth media. Hence, dissolved oxygen (DO) is a very important parameter for achieving high-density culture as well as for protein production. Oxygen has low solubility in water. We can improve the DO level in media by increasing the agitation speed, increased sparging rates, or by supply pure oxygen or air mixed oxygen into the media. Increasing the levels of DO from 30% to 300% saturation does not show any significant effect on the growth of *E.coli*, but may result in acetate accumulation and temporary decrease into respiration. In this study, the DO level setpoint was kept at 30% saturation. The 30% DO saturation was maintained by increased agitation speed and supply of pure oxygen with aseptic air in cascade mechanism into the bioreactor to support the growth & production of r-protein in *E.coli.*

c. pH: The optimal pH range for *E. coli* growth is 4.4- 9.2. *E. coli* metabolism displaces external pH to the extreme depending on the availability of substrates and nutrients in the environment. The pH also affects the culture conditions, the growth phase, and the composition of the metabolite. PH affects cell growth and low cell growth occurs at low pH. Many enzymes involved in acetate metabolism are induced at extreme pH. Maintaining the pH between6.5-7.0 reduces the formation of acetate by the cells in a medium due to which cell growth and protein production are not hindered.

d. Nutrient composition: The productivity of protein is associated with the concentration of cells present in the medium. Thus, for growing the cell culture to a large concentration for producing high titer of protein, choosing appropriate growth media is very crucial. As like other cells, *E. coli* also requires complete nutritional media which includes carbon source(s), nitrogen source(s), and sources of trace nutrients such as magnesium, sodium, potassium, sulfur, copper, zinc, etc., vitamins and amino acids for its optimum growth & survival(Decker et al, 2016). Some researchers used batch fermentation in that they used glucose mineral salt media to grow cells at high densities and they obtained various gm of biomass as shown in table 1.(Wolf-Dieter Deckwer et al, 1991). Other researchers used fed-batch fermentation in that they also used glucose mineral salt media with the addition of salts or carbon source. The results are as shown in table 2.(Wolf-Dieter Deckwer et al, 1991). There is a need for optimization and formulation of the media composition for the growth of *E.coli* because it can affect the metabolism of cell growth as well as the production of r-protein. For eg., the translation of different mRNAs is influenced differently by changes in the culture medium and the temperature. Mostly complex, defined, and semi-defined media are used to obtain HCDC(Berger et al., 2011). But according to Zanet et al 1988, to get a high yield of product or to boost product production only semi-defined or complex media are necessary. Because cell growth is generally slow in chemically defined media and also produces low protein titers than complex media(Zanette et al., 1998). In general, Most of the medium used to culture *E. coli* with HCDC contains a carbon source such as glucose or glycerin, salts such as phosphate, Na, magnesium, K, and other minor trace elements(Huang et al., 2012)(Volontè et al., 2011). Sohoni et. al. 2015, mentions, complex media like LB (Luria Bertani broth), Terrific Broth (TB), 2YT, GYT, MBL, Super broth (SB), M9 salt media, and Embase Flo media for the enhanced expression of the protein. Sometimes, semi-complex media are also used for cultivating *E.coli* to a high concentration of biomass for a high amount of protein expression. Since LB media is used most commonly for *E.coli*, it can be used for the preparation of seed media and TB media can be used in the fed-batch fermentation process in a controlled system i.e. Bioreactor for achieving high-density culture (Sohoni et al., 2015).

Table 1: List of researchers who used glucose mineral salt media to grow cell at high densities

Researcher name	Year	Biomass (gm/L)
Fieschko and Ritsch et al	1986	70
Shiloach and Bauer et al	1975	55
Bauer and Ziv et al	1976	68
Pan et al	1987	95
Cutayar and Poillon	1989	110

Table 2: List of researchers who used glucose mineral salt media with the addition of salts or carbon source to grow cell at high densities

Researcher name	Year	Biomass (gm/L)
Reiiing et al	1985	16.5
Kruger and Eppstein	1989	19
Mori et al	1979	38
Fass et al	1989	39
Gleiser and Bauer	1981	45

e. Induction strategy: Even though, the principle of HCDC leads to high-level expression of protein per volume of the reactor, the induction strategy for the protein expression should be optimized. During fermentation of recombinant organisms, the productivity of protein is optimum when the growth phase and induction phases are separate. After induction, the gene expression possesses a metabolic burden on the growing cells by channelizing most of the energy for the manufacture of recombinant protein. Thus, there is a need for optimization of the induction time, so that the metabolic burden due to the inducer affects the minimum on the growth of cells and protein production. Also, the inducer concentration should be optimized so that, it completely de-repress the gene and starts the expression of the gene(Panda et al, 2003). Kim et al 2007, have mentioned the use of lactose as an inducer molecule for the production of r-protein in *E.coli*. IPTG as an inducer molecule for the mass production of therapeutic protein is unsafe due to its non-metabolizable properties. Also, IPTG is expensive and sometimes it can be toxic. An alternative for IPTG islactose which has several benefits over IPTG such as 1. It can be hydrolyzed 2. Cheap 3. Safe for mass production of therapeutic proteins. Since most of the lactose is utilized by the *E.coli* cells, the cell concentration and expression levels were increased by 20% and 24%. M. Kim et al., 2007 has also mentioned getting the 20-fold higher expression of recombinant proilin by inducing the recombinant *E.coli* culture with lactose as compared to IPTG (Kim et al., 2007).

7. CONCLUSION:

The biopharmaceuticals industry is increasing day by day due to products that are available in the market. These products are majorly recombinant proteins. *E. coli* continues to be the ideal host for the large-scale production and expression of recombinant protein. In this review, the production strategies, role of r-DNA technology, different host and expression system for the production of recombinant proteins are discussed. In this study, role of high cell density fermentation for getting higher yield and productivity of recombinant proteins, effect of different parameters on high cell density fermentation, feeding strategy, optimization of medium for achieving higher growth are also discussed. Therefore, it can be concluded that the literature survey has been done on different criteria which needs to focus during high cell density fermentation of *E.coli* for the production of recombinant proteins.

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