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A BRIEF REVIEW ON DEVELOPMENT OF HIGH YIELD PROCESS IN ESCHERICHIA COLI FOR PRODUCTION OF RECOMBINANT CARRIER PROTEIN (CRM_{197})

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ABSTRACT

It is a well-established fact that bacterial polysaccharide – protein conjugate vaccine have made a huge impact on pediatric vaccination approach. The immunogenicity of polysaccharide is enhanced by coupling them to carrier proteins. The widely used carrier proteins are tetanus toxoid (TT), diphtheria toxoid (DT) and diphtheria toxoid variant CRM_{197}. DT conjugates are less immunogenic, TT conjugates renders reduced polysaccharide response whereas CRM_{197} are at lower risk for this.

Cross Reacting Material (CRM_{197}), a non-toxic variant of diphtheria toxin. Conventionally, CRM_{197} is isolated by fermentation of *Corynebacterium diphtheriae* C7 (β197) cultures, which often suffers from low yield. Several recombinant approaches have been reported with robust processes and higher yields, which will improve the affordability of CRM_{197}-based vaccines.

Escherichia coli based recombinant CRM_{197} carrier protein can be a revolutionary approach to get low cost and high yield carrier protein to serve the need of time and fulfill the demand of ever rising population of the world.

KEYWORDS: CRM_{197}, conjugate vaccine, carrier proteins, *Escherichia coli*.

1. INTRODUCTION

Infectious diseases have always been a scourge for humans and responsible for approximately 25% of global mortality, especially in children younger than five years (Kieny, 2004). Nowadays, modern technologies provide many opportunities to prevent infectious diseases by vaccination, which mainly capitalizes the immune system's ability to respond rapidly to pathogenic microorganisms upon a second encounter.

Vaccines have been described as 'weapons of mass protection' (Cohen and Marshall, 2001; Curtiss 2002). The goal of a vaccine is to induce durable immunological protection, whereby the first encounter with a pathogen is 'remembered' by the immune system. Therefore, the important step in the rational design of a vaccine is to understand the immune correlates of protection. From a mechanistic perspective, vaccines select, activate and expand memory B and T cells, which are then poised to respond rapidly and specifically to subsequent exposure of the pathogen. Today, prevention of bacterial and viral infections through vaccination is beneficial in reducing disease morbidity and health care costs.

There are wide varieties of vaccines available today which can be categorized into following main types: Live attenuated vaccines, Killed whole organisms, Subunit vaccines, Recombinant proteins, Synthetic peptides, Polysaccharides, and new generation polysaccharide conjugate vaccines also called as Glycoconjugate vaccines. It has been demonstrated that the polysaccharides are T cell independent antigen and not able to form Memory B cells and are poorly immunogenic. To overcome this issue, polysaccharides are conjugated with a nontoxic carrier protein to enhance the immunogenicity.

E. coli is a well characterized, safe (nonpathogenic) and suitable host for large scale production of recombinant proteins in a simple culture condition and lesser amount of time with respect to classical fermentation (Yee L et al., 1992; Kim et al., 2002; Akesson M et al., 2001). High-level expression of recombinant protein in *E. coli*

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often results in accumulating them as insoluble aggregates which can be purified easily with the available refolding, solubilization and chromatographic techniques (Singh and Panda, 2005).
CRM_{197} is classically purified from culture supernatant of the *C. diphtheriae* C7 (β197) tox negative (Tox-) strain (Uchida et al., 1973). The production of CRM_{197} from its C7 strain often suffers from low yield and also require sophisticated laboratory conditions to cultivate *C. diphtheriae* C7 strain. More recently, CRM_{197} has been produced in heterologous recombinant systems such as *E. coli* and *Pseudomonas fluorescens* (Patkar, 2012; Goel et al., 2017; Stefan et al., 2011) with higher yield. The characterization of CRM_{197} protein produced from *C. diphtheriae* C7 strain has been published (Broker et al., 2011; Malito et al., 2012). However, limited data is available for CRM_{197} produced from *E. coli* based recombinant source. Also, there is a lack of information whether recombinant CRM_{197} produced from *E. coli* is structurally and immunologically similar to its incipient counterpart (C7 CRM_{197}).

The increasing global demand of polysaccharide conjugate vaccines against encapsulated bacterial pathogens highlights the need for high yield processes delivering well-characterized carrier protein that meets regulatory and safety requirements.

2. VACCINES AND IMMUNE SYSTEM

The successful introduction of vaccines against various childhood illnesses was the greatest success story of the 20th century. Vaccination has become one of the most powerful means to save lives by decreasing the mortality rate and increasing the health level of humans.
A Vaccine is a biological preparation that improves immunity to a particular disease and is considered Prophylactic as it is administered in a healthy individual to prevent diseases (Bonanni, 1999). Immunity to infectious disease can be achieved by **Active** or **Passive** immunization. In both, the case the immunity can be developed by either a natural process (transfer from mother to fetus or by the previous infection from an organism) or by artificial means (transfer of Antibodies or Vaccines).
Passive Immunization was first shown by Emil Von Behring and Hidesaburo Kitasato that antibodies elicited in one animal can be transferred to another animal by injecting its serum into the first animal. In nature, the preformed antibodies are transferred naturally from mother through the placenta to the developing fetus (Baxter, 2007).

The aim of active immunization is to provoke the immune system which is of the following two types: (1) Humoral response and (2) Cell mediated response.

Humoral response: It is based on B Lymphocytes. Antibodies are the effector immunoglobulin's (Ig- A class of proteins which are glycosylated) of the humoral immune response. They bind to an epitope (that region of an antigen that interacts specifically with the antibodies) of the antigen which can then be readily ingested by phagocytic cells. The Antigen-Antibody complex also called immune complex also activates the complement system.

Cell mediated response: It is based on T Lymphocytes: T helper cells and T Cytotoxic cells. T_H cells interact with antigen-MHC II (Major histocompatibility complex Class II) molecule complex present on the APC (Antigen Presenting Cells); it becomes activated and starts secreting Cytokines which in turn activates B cells, T_C cells, and phagocytic cells. B cells proliferate to become Plasma cells and Memory cells. Plasma cells secrete antibodies and Memory cells are responsible for remembering the antigen upon subsequent encounter of the same antigen inside the body. T_C cells recognize the antigen-MHC I molecule complex and proliferate to form Cytotoxic T lymphocytes (CTLs) and Memory cells. CTLs kill the foreign antigen (Singh, 2005).

3. VACCINES AND IMMUNIZATION

The history of vaccine and immunization begins in the year 1796 when a country doctor in England, Edward Jenner, attempted to prevent infectious disease by means of immunoprophylaxis which involved the process of inoculation of pus scrapped from Cowpox blisters into an eight-year-old boy. The boy was then protected against the disease once he was challenged with pox material. The worldwide implementation of vaccination campaigns against smallpox allowed the WHO to declare its abolition in 1980(Fenner et al., 1988).
In the 19th century, Enders, Weller, and Robbins made a revolutionary discovery that the cells could be grown *in vitro* and used as a substrate for viral growth (both Polio and Measles). This strategy was immediately taken up

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by Vaccine developers (Plotkin, 2014). By the end of the 19th century, it was discovered that the immunogenicity could be retained by the bacteria if they were carefully killed by heat or chemical treatment. The 1st inactivated vaccines were developed by Salmon and Smith in the United States and the Pasteur Institute group (Roux and Chamberland) in France (Salmon and Smith, 1886). In 1970, the two discoveries i.e. the proteins can be expressed in the plasmid and that the DNA can be sequenced, started the era of genetic engineering. In 1986, these techniques helped to develop first recombinant Hepatitis B vaccine. In 1987, the application of conjugation led to a highly effective vaccine against *Haemophilus influenzae* type b which is the causative agent of Meningitis in children (Stevens et al., 1987).

With the advancement in vaccine technology, the production methods of vaccines have also advanced which has led to vast varieties of safe and highly effective vaccines. Modern industries are utilizing a wide array of currently available technologies to produce much safer and efficacious vaccines which include: viral vectors cultured in animal cells, virus-like particles cultured in yeast or insect cells, conjugation of polysaccharide to carrier proteins, DNA plasmids expressed and propagated in *E. coli*. Purification advances (e.g., Column based immune chromatography, precipitation, TFF techniques) have led to increase in efficiency for the purification and recovery of antigens whereas advancement in analytical methods (e.g., Dionex, MALLS, RNA microarrays) have improved the process understanding for the characterization of antigens(Josephsberg and Buckland, 2012).

4. TYPES OF VACCINES

There are various factors which are kept in mind while developing a safe and effective vaccine. The First factor is which branch of the immune system is activated after vaccination because development of immune response does not promises that the state of protective immunity has been achieved as there is a difference between activation of Humoral and Cell-mediated response. The second factor is the development of memory cells after vaccination, failing to do so will result in multiple doses of the vaccine.

This section describes different types of vaccines and the branch of the immune system that the vaccine invokes. Attenuated vaccines contain microorganisms (virus or bacteria) which have been attenuated or weakened so that they lose their ability to cause significant disease but retains their ability to transient growth in the vaccinated individual. Attenuation can be achieved by culturing the pathogen for a prolonged time under abnormal conditions.

Many attenuated Viral {Measles, Mumps, Rubella, Yellow fever, Polio (Sabin)} and Bacterial (Tuberculosis, Typhoid) vaccines have been in successful use. An attenuated strain of *Mycobacterium bovis* called BCG (Bacillus Calmette Guerin) was developed on media containing an increasing concentration of bile. Poliovirus used in Sabin vaccine was attenuated by growth in monkey kidney epithelial cells (Minor, 2015).
The advantage of an attenuated vaccine is that they are capable of transient growth which allows prolonged exposure to the immune system which results in increased immunogenicity and produces memory cells as a consequence these vaccines often requires only a single dose.
The major disadvantage of the attenuated vaccine is the possibility of the attenuated strain to revert back to its virulent form.

Inactivated vaccines are made from microorganisms (viruses, bacteria, other) that have been killed through heat or chemical treatment which cannot cause disease. It is important to maintain the structure of epitope of antigen during inactivation. They mainly provoke the humoral branch of the immune system. Whole cell pertussis and Salk Polio vaccine are produced by formaldehyde inactivation.
The advantage of an inactivated vaccine is that it avoids the risk of live organisms. The major disadvantage of these vaccines is that they require multiple booster doses to maintain the immune state of the host(WHO vaccine safety training / module 2).

To avoid the risk of the whole organism as a vaccine, purified macromolecules from the concerned pathogens are used as a safer option for vaccine development. Three general forms of such vaccines are presently in use: (1) Capsular polysaccharide (2) Inactivated Exotoxins (3) Recombinant surface antigens (Shenoy, 2007).
Capsular polysaccharide: These vaccines are based on the fact that the virulence of certain pathogenic bacteria's is based on their hydrophilic polysaccharide antiphagocytic capsule. These types of vaccines invoke

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the opsonizing antibodies and are administered in high-risk individuals like infants, elderly and other immune suppressed individuals. Examples of such types of vaccines are: Typhoid, Paratyphoid, Pneumococcal, Meningitisetc. (Ni et al., 2017).

The disadvantage of capsular polysaccharide vaccine: It does not provide long- lasting immunity as it generates IgM antibodies instead of IgG antibodies in infants (T cell independent, no memory effect) and frequent vaccination can lead to low responsiveness.

Inactivated Exotoxins: Some pathogenic bacteria produce exotoxins which is responsible for their virulence nature (Diphtheria and Tetanus). These exotoxins can be inactivated by its treatment with formaldehyde to form toxoid which is used as a vaccine. Vaccination with toxoid induces specific antitoxin IgG antibodies. Diphtheria toxoid combined with tetanus and pertussis vaccines (DTP) has been part of the WHO Expanded Program on Immunization (EPI) since its inception in 1974 (WHO Position paper, 2018).
Recombinant antigen vaccine: Any gene that encodes a protein can be cloned and expressed in bacterial, yeast, insect or mammalian expression system using recombinant DNA technology. In 1986, this technique was used to develop the first recombinant Hepatitis B vaccine (Stevens et al., 1987). The recombinant *Pichia pastoris* cells are cultured in production fermentor where the HBsAg (Hepatitis B Surface antigen) is expressed intracellularly. First, the cells are ruptured mechanically and chemically to get the desired antigen in the broth followed by several purification techniques like chromatography, tangential flow filtration, and centrifugation etc. are used to recover purified HBsAg antigen.

Multivalent Subunit vaccines: As the name indicates this type of vaccine contains multiple types of polypeptides or multiple copies of a particular polypeptide in a single vaccine.

Solid matrix Antigen-Antibody complex (SMAA) is prepared by attaching monoclonal antibodies to a solid matrix and then saturating the antibodies with desired polypeptide antigens which have epitope for both T and B cells to evoke both cellular and humoral immune system. Another example of multivalent subunit vaccine is ISCOM or the Immuno Stimulating Complex which can be prepared by mixing the antigen and glycoside called Quil A (Kubey, Immunology 7th Edition, 2013).

In another approach of multivalent subunit vaccines, a vaccine was formulated against a human pathogen *Francisella tularensisis* which is a causative agent for disease Tularemia. The formulation contained a combination of *F. tularensis* protecting antigens: OmpA-like protein (OmpA), chaperone protein and lipoprotein from the highly virulent *F. tularensis* SchuS4 strain (Banik et al., 2015).

DNA vaccines have provided a new platform to immunize an individual with a recombinant plasmid encoding desire gene for antigenic protein. The injected plasmid is taken up by the muscle cells and transcribed. The resulting antigenic protein invokes both humoral antibody and cell-mediated response. The plasmid DNA is coated with microscopic gold or tungsten particles and then delivered into muscle with gene gun using compressed helium as the accelerator. Recently a plasmid was developed which contained full-length genomic RNA sequence of live attenuated alphaviruses and flaviviruses which when administered *in vivo* gets transcribed to induce partial replication of a genetically defined, live-attenuated vaccine virus in the tissues of the recipient, thereby inducing a protective immune response (Pushko et al., 2016).

Glycoconjugate Vaccines: Some serious bacterial infections are caused mainly by encapsulated bacteria's like *Haemophilus influenzae* type b (Hib), *Streptococcus pneumoniae*, *Neisseria meningitides*, and *Salmonella typhi*(Trotter et al., 2008). Glycoconjugate vaccines have been developed to overcome the limitations of polysaccharide vaccines which give rise to T cell independent immune response. These vaccines covalently conjugate polysaccharides from the encapsulated bacterial surface to a carrier protein. The protein portion is recognized by T cells (T cell-dependent immune response). In 1929, Avery and Goebel demonstrated the conjugation of sugar with protein which was able to induce antibody in an animal model (Avery et al., 1929). Between 1987 and 1990, the first conjugate vaccine against *Haemophilus influenzae* type b (Hib) was licensed (Schneerson et al., 1980).

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Polysaccharide-protein conjugate is processed in endo-lysosome of Antigen presenting cells (APCs) as a result of which a polysaccharide specific epitope is generated on the surface of the APCs. The polysaccharide specific epitope along with carrier protein-derived peptide binds to Major Histocompatibility Complex II (MHC II) and stimulates T_H cells to produce Interleukins 2 and 4. As a consequence, a conjugate immunizing agent induces a T-cell-dependent response already early in life that ends up in immunologic memory and boosting of the response by more doses of the vaccine (Costantino et al., 2011).

5. CARRIER PROTEINS

There are 5 carrier proteins that are in use for production of Glycoconjugate vaccines: a genetically modified cross-reacting material (CRM) of diphtheria toxin, tetanus toxoid (TT), meningococcal outer membrane protein complex (OMPC), diphtheria toxoid (DT), and *H. influenzae* protein D (HiD). All the 5 carrier proteins are highly effective in increasing the immunogenicity of the vaccines (Pichichero, 2013).

CRM_{197} is genetically modified and detoxified variant of diphtheria toxin isolated from *Corynebacterium diphtheriae* C7 (β197). A point mutation at amino acid positioned at number 52 replaces glycine with glutamic acid as a result of which ADP-ribosyl transferase activity of the protein toxin is lost. It is a single polypeptide of 535 amino acid with a molecular size of 58.4 kD and has two subunits (fragment A (catalytic domain) and fragment B (transmembrane domain) linked with disulfide bridge (Malito et al., 2012). It is antigenically indistinguishable from diphtheria toxin and has more lysyl side chain for conjugation reaction. CRM_{197} also has binding site for EGF (Epidermal Growth factor) receptor which is mainly overexpressed on tumor cells, as a result, many scientists are working on using CRM_{197} as anti-tumor therapy (Buzzi et al., 2004).The receptor binding subdomain of B domain has β-sheet structure whereas the remaining subdomain of B domain has nine α-helices (Mishra et al., 2018) Currently CRM_{197} is manufactured in *Corynebacterium diphtheriae* C7 strain or from a plasmid system in *Pseudomonas fluorescens*. The maximum achievable yield in either of the system is very low and their production requires a Biosafety level II facility which further adds up in the cost of the vaccine (Blattner et al., 2017). For the production of Glycoconjugate vaccine, polysaccharide has to be activated i.e. chemically modified. Activation can be achieved by various available methods like Reductive amination (an active aldehyde group is created between adjacent carbon having –OH group), Cyanylation (active –CN groups are created randomly by changing –OH groups) and Carbodiimide (–OH of a carboxyl group is replaced by Carbodiimide group). Once the polysaccharide has been activated, it can be chemically linked to a protein (Frasch, 2009). To achieve a higher yield of CRM_{197}, the fermentation process poses a big challenge and requires a lot of optimization; as a result, lots of development studies are underway to use bacterial hosts as an alternate to *Corynebacterium diphtheriae*. Many attempts have been made to develop CRM_{197} in heterologous recombinant systems like *E. coli* (Stefan et al., 2011). However, there is very less data available on the characterization of the recombinant CRM_{197} produced from *E. coli* and also lacks the information about its structural and immunological similarity with the native CRM_{197} produced from C7 strain (Hickey et al., 2018). Hence, the successful expression and purification of the whole CRM197 protein in *E. coli* have not been reported.

Since the global demand for conjugate vaccines against the deadly bacterial diseases is increasing, there is a pressing need to develop a process which meets the demand of high yield, lower cost, and a carrier protein which meets the safety and regulatory requirements.

6. *ESCHERICHIA COLI* AS A MODEL ORGANISM

E. coli also known as "molecular biologist tool box" is the most widely studied and well-characterized microorganism in the history. It was discovered by Theodor Escherich, in 1885. It is also called as "workhorse" of molecular biology for its fast growing rate in chemically defined media. It has a long history of laboratory culture and ease of manipulation. There is a vast knowledge available on *E. coli* for its genetic makeup, genomics, transcriptome, proteome, and metabolome (Idalia and Bernardo, 2017). Many strains of *E. coli* are considered as Biosafety level I organism which can be handled in a normal laboratory and does not require a special classified area for its handling. The foundation of biotechnology was led by pioneering work of Stanley Norman Cohen and Herbert Boyer in *E. coli* who created a recombinant DNA molecule by using plasmid DNA and restriction enzymes.

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*E.coli*is a gram-negative, rod-shaped, facultative anaerobic, of the genus Escherichia. It can be grown on simple laboratory settings and inexpensive media. It is a chemoheterotrophic organism which requires a source of carbon and energy in its culture media (Russo, 2003). *E. coli* is considered as a host of choice for DNA cloning since cloning and isolation of DNA insert is easiest in *E. coli*. Strain K12 of *E. coli*is most widely used and it supports several types of vectors such as plasmid, bacteriophage, cosmids, shuttle vectors etc. It also has a circular plasmid other than its bacterial chromosome and is capable of independent existence. This natural plasmid of *E. coli* has been modified, shortened, reconstructed and recombined to create plasmids of enhanced utility and functions (Lara et al., 2012). The gene of interest is isolated and amplified by inserting the gene into a plasmid with the help of restriction endonucleases and DNA ligases. The cloned gene is then expressed into the host organism where the gene of interest is transcribed and translated to produce the protein encoded by the inserted gene. *E. coli* is used both for cloning as well as expression of the gene as host organism (Stryjewska et al., 2013).

7. BIOPHARMACEUTICAL MANUFACTURING PROCESS

Process development journey: The journey that a therapeutic drug travels from research and development to commercial manufacturing is a long and tedious journey.

Research and development

The journey of a biopharmaceutical product starts with the **development program** where all the basic research like strain development, strain improvement, process development, identification of CQA (Critical Quality Attributes), CPP (Critical Process parameters), KPP (Key Process parameters) etc. is performed (PDA, technical report no. 60, Process Validation: A lifecycle approach). Once a product is formulated, **preclinical phase** commences, it included testing the drug for its pharmacokinetic, pharmacodynamics properties, dose effect, and toxicity properties. An animal model equivalent to human has to be identified i.e. initially in lower species like rodents or mice and finally, the drug is trialed in non-human primates (NHP) such as rhesus or cynomolgus monkeys (Mundae and Östör, 2009). After completion of the preclinical phase, the drug enters the clinical phase which involves testing the drug in humans and is carried out in three phases. **Clinical phase I** involves testing the drug in a small number of people (40-60) for its safety and possible side effects and to evaluate the route of the drug administration. **Clinical phase II** involves more number of people than phase I trial (20-300) to check the safety and efficacy of the drug. **Clinical phase III** is a confirmatory study involving from 300 to 3000 subjects for safety and efficacy. It also includes a comparison of a drug with standard treatment. After successful completion of all the phases of clinical trials, a drug is allowed by the regulatory authorities for commercial manufacturing. **Clinical phase IV** is post-marketing surveillance to record any adverse effect or toxicity that may have been caused by thedrug(https://www.novartisclinicaltrials.com/TrialConnectWeb/whatisclinicaltrials.nov).

Fermentation: During manufacturing of a biological product, it is very critical to have a common starting source for each production lot i.e. having a well characterized and preserved cell bank. The practice of two-tiered cell banking system is the most practical approach in which a working cell bank (WCB) is produced from the master cell bank (MCB). WCB serves as the supply source of cell substrate for the continuous manufacturing of the substrate (ICH Q5D, version 4, 1997). The preserved WCB (Glycerol stock at -80ºC, lyophilized cell banks etc.) is then revived and inoculated either on media plates or culture flask which serves as inoculum for large scale cultivation of culture.

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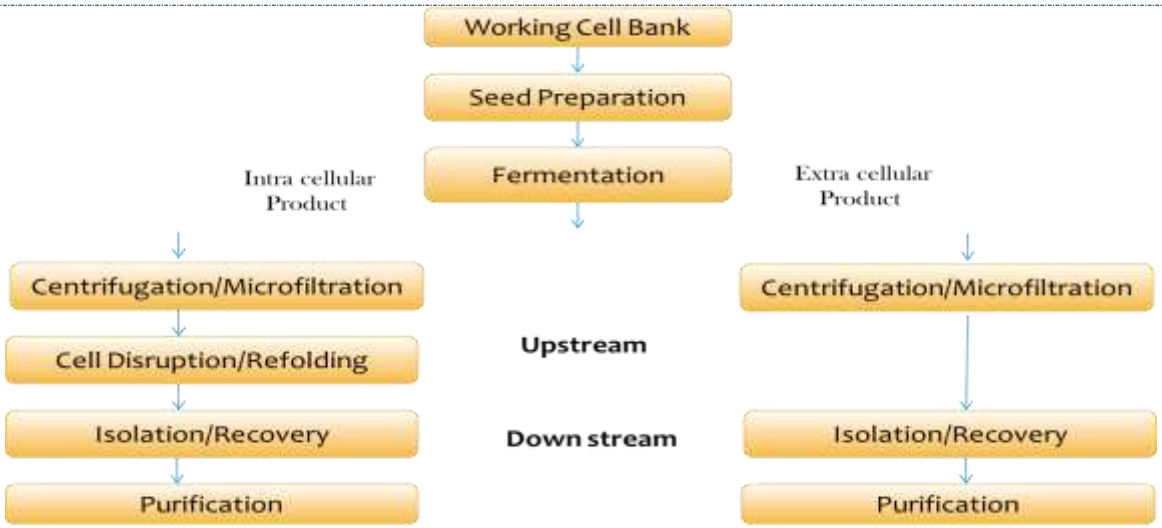

Figure 4: Process flow of Microbial/Yeast Fermentation

Once the culture has reached its optimum growth/production level, the culture broth is harvested and concentrated with different techniques like centrifugation, microfiltration, tangential flow filtration etc. If the product of interest is intracellular, it involves cellular disruption step or else the supernatant is processed using various precipitation, chromatography, filtration steps to recover the purified product of interest.

For *E. coli*, and other fermentation system, the accumulation of intracellular recombinant protein is dependent on the final cell density and the specific activity of the protein. Usually, there are four different strategies adopted for optimizing the production of a recombinant protein, like expression system control, strain improvement, optimization of culture medium and mode of fermentation (Broedel et al., 2001).Fermentation can be carried out in 3 different modes i.e. batch, fed-batch, and continuous fermentation. In batch mode, all the nutrients and media components are added initially and then the batch is harvested after completion of growth. In acontinuous mode of operation the nutrients are continuously added to the fermentor and at the same time product is also continuously withdrawn at the same rate so that the volume of fermentor remains constant. Fed-batch process falls between these two process where the initial nutrients present in the fermentor leads the batch to a certain growth limit and later the growth is increased by subsequent addition of nutrients. The product is harvested at the end of the batch. Fed-batch processes are often used for achieving high cell density and thereby improving productivity. During fermentation of *E. coli*, it is very often that growth inhibitory acetate accumulates in the culture if the concentration of carbon source (usually glucose) is not controlled. Production of acetate can be minimized by fed-batch technique where glucose and other nutrients can be supplied at a controlled rate. The yield of recombinant product is dependent on both the biomass concentration and the cultivation condition (Yee and Blanch, 1992).

The development of a high yielding process will reduce the ever rising demand of the carrier protein which is often considered as the bottleneck for the development of Glycoconjugate vaccines and these vaccines can be made available to the common people at a lesser price.

Conclusion

Production of cross-reacting material (CRM) from *Corynebacterium diphtheria* does not provide a high yield to meet the ever-rising demand of Glycoconjugate vaccines.

By optimization of fermentation techniques and the vast historical knowledge available about culturing of *E. coli*, high-level production of recombinant CRM_{197} protein can be achieved which will result in the availability of cost-effective Glycoconjugate vaccines (Typhoid-CRM, Paratyphoid-CRM, Pneumo-CRM etc.)

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