Benefits and Biohazards of Microbial Recombinants

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Abstract

Recombinant DNA technology has brought paradigm changes in the field of biological interventions in modern society. Be it pharmaceuticals, nutraceuticals, or other products of commercial use, recombinant proteins have brought new opportunities. Way back in 1974, a paper in the journal *Science*, commented on the acquisition of new capabilities in recombinant DNA (rDNA) technology and the immense opportunities in biological sciences created thereby. At the same time, potential biohazards of the technology were also pointed out, which had earlier been discussed by a group of scientists in the 1973 Gordon Research Conference. The scientists called for a voluntary deference of experimentation using rDNA technology. Today, strict guidelines exist regarding the use of rDNA technology for basic and applied sciences. This chapter discusses the various benefits as well as biohazards of microbial recombinants, i.e., rDNA technology using microbial strains and viral vectors. The known and upcoming recombinant products are documented, along with a discussion on the biohazard issues.

Keywords

rDNA technology · Microbial recombinants · Genetic engineering · Protein purification · Biohazard

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9.1 Benefits of Recombination: The First Recombinant

Utilization of the basic process of recombination for expression of desired proteins in simple organisms like *Escherichia coli* opened new vistas in bio-based industries. The first sector to use this technology was the pharmaceutical industry, which designed engineered insulin protein(s) for management of diabetes (Quianzon and Cheikh 2012). rDNA technology helped design new pharmaceuticals with desired biological activities. But before the advent of rDNA technology, insulin derived from animals was the only product available for treating diabetes. For the discovery of insulin and establishment of its blood glucose reduction effects, Frederick Banting, Charles Best, and John MacLeod got the Nobel Prize. The story of pharmaceutical insulin aptly explains the enhancement of research repertoire, achieved by rDNA technology (Quianzon and Cheikh 2012). The company Eli Lilly started insulin production from animal pancreas, but challenges of various dimensions were encountered soon. Due to the use of animals, meeting the demand was an issue. Potency of the product varied to levels as high as 25%. Animal insulin also suffered from the drawback of allergic reactions and toxicities in humans. Later years saw improvements in methodology, incorporating protamine and zinc to improve peak activities and further refinement in purification. It was in 1978 that Goeddel et al. expressed the two chains A and B of human insulin in *E. coli* to produce rDNA insulin. Genentech and Eli Lilly joined hands to commercially produce rDNA insulin, named Humulin. Two versions R (rapid) and N (intermediate-acting) were produced and marketed.

Cloning of the insulin gene and its successful production opened newer ways for modification to further upgrade the product. Site-directed mutagenesis of key amino acids in the insulin protein was used to achieve desired pharmacokinetics, absorption level, peak, and duration of action. This led to more products in the market, namely, lispro, aspart, glulisine, glargine, detemir, etc. rDNA insulin is a wonderful example that demonstrates addressing of challenges using nonconventional tools. Talking to The New York Times at the announcement of approval of Humulin on 30th October 1982, Dr. Henry Miller, the medical officer in charge of Humulin at the FDA, commented that the development of Humulin was a major step forward in the scientific and commercial viability of recombinant DNA techniques and that "We have now come of age," referring to the dawn of new tools in the hands of medical research community to offer required products and solve existing challenges (http://members.tripod.com/diabetics_world/humulin_history.html). Thus, cloning and successful expression of a human protein in a simple bacterium not only upscaled production but also offered avenues of further improvements in the basic product and its quality control.

9.2 Overview of the Technology and Advantage with Microbial Hosts

The immense potential inherent in rDNA technology is mainly because of the ability to join/delink/modify DNA. The specific advantage with microbial recombinants is the flexibility offered due to simplicity of prokaryotic organisms. *E. coli* has been successfully used as an experimental organism (Cronan 2014). *E. coli*, also known as the workhorse of molecular biology, has simple nutrient requirements and a rapid generation time of 18 min, which brings both cost- and time-effectiveness during expression of a desired protein. Protein expression in any host utilizes the basic fundamental of molecular biology, also referred to as central dogma, which outlines the flow of information in cellular systems (Fig. 9.1a). DNA, being the defining molecule, is used for directing the microorganism to produce a particular protein

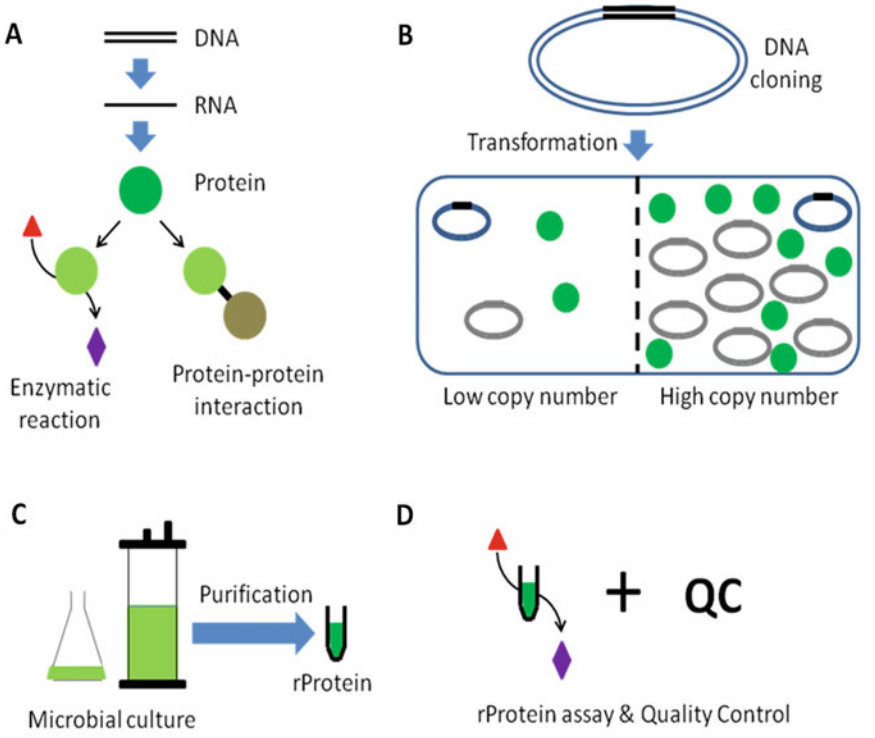

Fig. 9.1 Overview of rDNA technology in microbial hosts. (**a**) Central dogma of molecular biology. (**b**) Cloning of the DNA encoding a desired protein in plasmid and transformation of the plasmid in a compatible microbial host. Depending on the stringency/regulations of application of a particular protein, a choice is made to use either a low- or high-copy-number plasmid, among other factors. (**c**) Protein expression in culture flasks or fermentor based on scale of production and its downstream processing to yield recombinant protein purified to homogeneity. (**d**) Determination of activity of the recombinant product and its quality control

(Fig. 9.1b). Many factors like codon usage, choice of promoter to drive gene expression, and type of plasmid in terms of copy number to be used for protein expression need to be optimized for successful protein expression (Rosano and Ceccarelli 2014). For example, while high-copy-number plasmids warrant more protein expression, the same may also result in mutations. This situation can be avoided by using low-copy-number plasmids. In practical scenario, the choice would be determined in part by the type and usage of protein being expressed and the effect of mutations on it. In addition to the many tools for manipulating DNA, a number of expression systems have been characterized and standardized in model microorganisms like *E. coli, Saccharomyces cerevisiae, Pichia pastoris*, etc. Inducible expression systems have been designed for cloning of proteins that are toxic to the host. Advances in fermentation technology and downstream processing have resulted in optimized protein purification protocols capable of purifying desired protein to homogeneity (Fig. 9.1c). The purified protein is finally tested for its activity and other quality control parameters (Fig. 9.1d).

Many factors influence the choice of microbial host system, which include the necessity of posttranslational modifications like glycosylation, secretion of the overexpressed protein in media for easier purification, etc. Although many advances have been made in insect cell and mammalian cell cultures and they have proved their potential as upcoming expression platforms, microbial recombinants still top in terms of number of products made in them. *E. coli* continues to enjoy its position as the topmost protein expression platform in terms of number of pharmaceuticals (Sanchez-Garcia et al. 2016).

9.3 Applications of Recombinant Proteins in Medical and Nonmedical Industry

While most of the recombinant pharmaceuticals have been designed for treating metabolic disorders, products for treating hematological disorders and cancers also abound (Sanchez-Garcia et al. 2016). Prokaryotic microbial host *E. coli* and eukaryotic microbial host *S. cerevisiae* together express around three-fourths of the total recombinant proteins. Many products like Protropin, Roferon A, Intron A, Recombivax, Humatrope, filgrastim, pegfilgrastim, and many antitumor drugs have been expressed in micro-organisms. This indicates the utilization of microbial recombinants in medical sector.

Similar to changes in the pharmaceutical paradigm brought, new markets have resulted due to increased opportunities in rDNA technology. An example of creation of a new market is the fully vegetarian cheese and dairy products. Many populations of the world stringently follow vegetarianism. In dairy industry, rennin was widely used for cheese making. Rennin was isolated from young calves. Nowadays, microbial strains are used to express recombinant coagulants. Many companies have advertised this strategy in order to accomplish better sales from the customer base concerned for vegetarianism (https://economictimes.indiatimes.com/amuls-worlds-biggest-vegetarian-cheese-brand/articleshow/1288927.cms). Other important

products include those needed in cosmetics (Sunar et al. 2016), detergent industry (Wang et al. 2018; Fariha et al. 2010), animal feed and additives industry (Claudia et al. 2013), food industry (Claudia et al. 2013), and vitamin industry (Jose-Luis and Arnold 2010). The portfolio of industrial proteins is wide and includes phytase, laccase, proteases, antibiotics, lipases, etc., tailored to various applications. Cellulase and β-glucosidase have been manufactured for biofuel industry (Puetz and Wurm 2019). For nonmedical applications, protein function is of utmost function, while for those intended for medical use, both protein function and its reception by human/animal immune system assume significance.

9.4 Viral Vectors and Applications

Viruses have the natural ability of introducing DNA into specific host cells. They have been modified for use in recombinant DNA technology. Viral vectors have gained much importance as they are widely used as tool for delivery of genetic material in molecular biology experiments, both in vivo and in vitro. The process of gene transfer by viral vector is known as transduction, and this process was harnessed in molecular biology experiments in the late 1970s when modified form of SV40 viruses was used to infect in vitro cultures of monkey kidney cell lines (Vanncci et al. 2013).

The major aim behind development of viral vector was to increase the probability of DNA uptake by host cells without affecting viability and viral vector proved to be good alternative for gene delivery as compared to traditional methods of DNA uptake by chemical treatment. The added benefit of using viral vector is that certain viral vectors integrate the delivered DNA into host cell genome which ensures stable expression of transferred gene. Advantages associated with viral vectors have increased their applicability in basic molecular biology experiments as well as clinical studies, which gives avenues for construction of novel viruses used in gene therapy and for vaccine development (Perricaudet and Stratford-Perricaudet 2012).

There are two broad categories of viral vectors, depending on whether their genome integrates into host chromatin or remains in the nucleus majorly in the form of extrachromosomal episomes. Lentiviruses and oncoretroviruses are the two subgroups which integrate their genome into host DNA, whereas adenoviruses, adeno-associated viruses, and herpes viruses come under the category of vectors that exist as independent entity in the host nucleus. The aforementioned classification is important to ascribe the applicability of viral vector for a specific purpose. For example, integrating viral vectors can be used for the dividing host cell, whereas non-integrating vectors are of major choice when dealing with non-proliferating stable cell lines. As there is no single multipurpose viral vector available, there is need to develop specific viral vectors as per demand of a particular target (Bouard et al. 2009). The choice of a particular viral vector is established by several parameters which include stability constraints, production processes, demand of

their transient or long-term expression, and the need of regulating the transgene expression.

9.5 Biohazards of Microbial Recombinants

One of the initial concerns about the nature of microbial recombinants was expressed by the Committee on Recombinant DNA molecules (Berg et al. 1974). While the technology has come a long way since then and many products have been commercialized, still combating the biological obstacles posed by cell factories and standardization of methodology is a huge challenge for rDNA pharmaceuticals in the development of protein-based molecular medicine. The major reason for some of apprehensions regarding the recombinant enzymes and pharmaceuticals from the market is low product quality, which is a common problem associated with biological synthesis of target proteins in host systems (Ferrer-Miralles et al. 2009). Poor protein quality of expressed recombinant protein in microbial cell factories is mainly due to altered folding, changes in codon preference, formation of inclusion bodies, and triggering of the consequent cell responses in host cell (Gasser et al. 2008). A reason for the potential hazardous nature of recombinant proteins is distortion in the protein structure, also known as misfolding. At the atomic level, a protein is composed of secondary structure elements α-helices and β-sheets. The spatial arrangement of secondary elements imparts a unique three-dimensional conformation to the protein, which is important for its biological activity. In the case of protein enzymes, active site (where the enzymatic reaction takes place) and allosteric sites (which are influenced by cofactors) are important. In case of protein-protein interactions, hotspot regions whereby two or more proteins recognize each other to manifest biological functions are essential. Any changes at the important sites that disrupt the normal activity will render the protein ineffective. In case of pharmaceutical proteins, misfolding of protein structure has the additional drawback of possibility of immunological reactions, in case the misfolded site adopts a local conformation recognized as "foreign" by the body's immune system. The recombinant proteins used presently for the treatment of several human disorders, including malignancies, endocrine, infectious, inflammatory diseases, etc., are similar to naturally occurring human proteins. In human body, these recombinant proteins prove to be immunogenic, which is a major setback for their frequent use in medicine development, as autoimmune response towards these self-antigens is observed by the administration of these recombinant proteins. The antibody production represents a system response against an exogenously administrated recombinant drug which leads to unwanted immune response causing loss of efficacy, which distorts the ability to distinguish self from non-self-antigens and serious side effects. The major obstacle for developing stable recombinant drug is to analyze, characterize, and finally quantify the amount of anti-recombinant drug antibodies induced by it in patients, and further major challenge is to overcome these side effects. Figure 9.2 provides a schematic representation of the protein structure along with secondary structure elements.

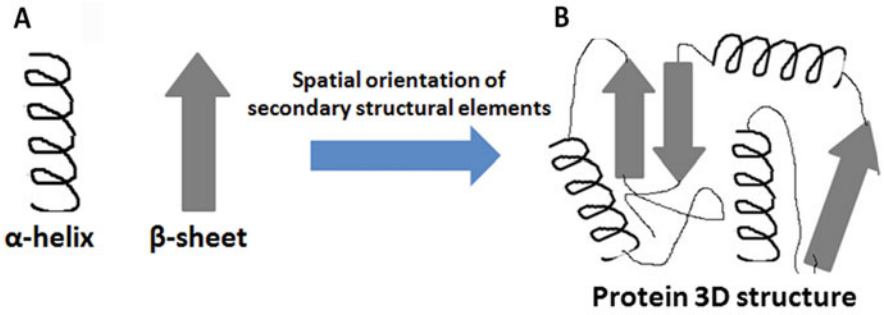

Fig. 9.2 Overview of the protein structure. (**a**) Secondary structure elements. (**b**) Spatial arrangement of secondary structural elements with loops that result in distinct three-dimensional conformation of a particular protein, important for its biological function

Hence, due to issues described above, recombinant proteins may suffer from certain post-application hazardous effects, some of which are described below:

1. **Hypersensitive Reactions Against Recombinant Protein in Host System**

With consistent use of recombinant pharmaceuticals, there are more evidences for induction of immune response in patients which seems to be a major issue not only during development of recombinant drugs but also during marketing these drugs after several clinical trials.

(a) *Insulin*

Hypersensitive reactions have been observed since early times due to the administration of insulin produced in beef or pork for treatment of type I diabetes, mainly due to antibody production. Anti-insulin antibody, particularly IgE, is ascribed to induce skin and anaphylactic reactions which necessitated increased insulin doses over time to gain its primary benefits, i.e., treatment of diabetes. rDNA technology came with vision of developing human insulin by gene technologies that was assumed to eliminate the immunogenic reactions, but such benefits were not fully attained as reports regarding insulin allergies to human insulin exist (Durand-Gonzalez et al. 2003). It was observed that amount of antibodies with cross-reactivity was greater when treatment was dependent on application of subparts of insulin protein compared to natural form of insulin, but clinically the information about these antibodies is obscure. The antibodies against insulin may potentially lead to autoimmune type I diabetes. The need of comparing the levels of antibodies against insulin before and after administrating insulin and correlating it with diabetes induction has been stressed.

(b) *Interferon*

Naturally occurring interferons have many defense-related benefits. For example, alpha-interferons are known to inhibit viral replication. Beta-interferon has been used to treat multiple sclerosis (MS) (Limmroth et al. 2011). Immunogenic reactions against these recombinant interferons have been observed due to the production of neutralizing antibodies (NAbs). Fibroblast interferon is the only beta-interferon present naturally in humans. The relapse rate of multiple sclerosis was observed to be reduced by the administration of recombinant beta-interferon named interferon-beta-ser (Betaferon®). Later on, it was observed that neutralizing antibody production was more in multiple sclerosis-affected patients administrated with (Betaferon®) essentially due to variation in the molecular domains of artificially produced interferon-beta-ser and its natural counterparts, i.e., beta-interferon (Limmroth et al. 2011). Earlier, it was reported that one-third of MS patients treated with recombinant interferon developed antibodies within the first year of therapy. In some cases, antibodies even appear to stimulate the function of interferon. Hence, it was recommended that treatment decisions should be made on case-to-case basis.

(c) *Erythropoietin*

Erythropoietin (Epo) is an activator protein for erythroid progenitor cells through receptors known as Epo-receptors. Epo mainly induces the erythroid progenitor cell for production of red blood cells. In normal person, the kidney is the target organ for production of erythropoietin that is produced in sufficient quantities by the kidneys under normal conditions, but due to renal failure erythropoietin content decreases drastically which subsequently affects RBC production through erythroid progenitor cells which generates anemic condition in affected patient (Fisher 2003). The treatment of erythropoietin deficiency anemia was treated using recombinant form of human erythropoietin known as r-HuEpo since the late 1980s, and it was found that r-HuEpo do not induce immunogenic reactions in in vivo systems as the recombinant erythropoietin had nearly identical protein structure as that of its naturally existing counterparts with minute difference in pattern of sugar moieties associated with protein part of erythropoietin (Skibeli et al. 2001). Recombinant erythropoietin was found to induce a disease condition known as pure red cell aplasia (PRCA) in rare cases at initial period of its use, and just three cases were reported until 1998 by Eckardt and Casadevall (2003). However, with time, the recombinant erythropoietin-mediated PRCA was increased in receptive patients of recombinant erythropoietin drugs due to generation of neutralizing antibodies (Casadevall et al. 2002). Thereafter, the number of patients diagnosed with PRCA has increased substantially (Verhelst et al. 2004). This data reveals regular need of monitoring the immunogenicity of recombinant proteins even after its approval for clinical use.

2. **Autoimmune Response and Pharmacokinetics**

Autoimmune antibodies mainly affect the immune-complex formation, hypersensitivity reactions, and pharmacokinetics. The reason behind production of autoimmune antibody is elevated subjection of unnatural epitopes to the immune system, newness of recombinant drugs to the host immune systems, the presence of adjuvants, varied carbohydrate patterns in glycosylated protein products, drug aggregate formation, the predisposition of genetic system to autoantibody formation, and the previous exposure of identical compounds in an individual immune system. Established examples of antibodies produced against recombinant drugs were antibodies against factors VIII and IX. The clinical management of hemophilia A and B is mainly managed by acting as inhibitors (Oldenburg et al. 2004). There should be prevention of antidrug antibodies which reduces benefit of a given drug and increases the risk of autoimmune reactions. Recombinant drug development comes with additional step of detecting and monitoring antibodies before, during, and after drug application through special clinical trials. There is need to develop highly specific assays and technologies to completely understand the immunogenicity of a particular recombinant protein in a particular person. The autoimmune status of a particular patient has to be included into drug development program for overall assessment of antibodies produced due to autoimmune response. The antidrug antibody production can be reduced by advancement in recombinant technologies, by establishing the underlined mechanism of autoimmunity, along with the role of human genetics and environmental factors that affects immunogenicity.

3. **Biohazards of Virus-Derived Vectors**

Viruses have a natural tendency of caring genetic material into the host cell and introducing there genetic material into host genome; this natural process has gained much importance in rDNA technologies by exploiting virus-based systems as a vector for transferring required genetic information in host cell. Virus vectors are formed by genetically modifying the natural viral genome by removing virulence sequences while keeping sequence important for gene delivery, integration, and functional effectiveness. Virus vectors differ on the basis of insert size, duration of gene expression, targeted host, and the wild-type virus from which they are derived. Clinical trials are conducted prior to approval of genetically modified organism as pharmaceutical products, so there is need to contemplate that a precise boundary exists between deliberate use and contained use of recombinant proteins that contained GMO, as patients who accrued recombinant drugs are in long-term exposure to natural environment which increases the risk of spreading these GMO in the environment.

Genetically modified viral vector used for medical benefits may add adverse effect to the environment due to alteration in the properties compared to the parental virus, such as changes in aspects of the viral life cycle, the viral vector interactions with host, change in the gene products encoded by the insert sequence within host system, increased virulence in comparison to the parental virus in the human

nontarget population, increased pathogenic effects including toxic and allergenic effects in animals, effects on the population dynamics in the natural environment, e.g., effects on the dynamics of populations of species in the receiving environment, and the genetic diversity of each of these populations (Baldo et al. 2013). The gene product expressed in virus vector may have intrinsic hazardous properties by acting toxic or allergenic in host system, and combating these hazardous effects in clinical trials solely depends on genetic and physiological context of parental virus, its interaction with host system, and condition of use.

Some of the biohazards associated with virus vectors are as follows:

(a) *Altered virulence*: Recombinant viral vectors can undergo inadequate virulence changes which increase the risk to both environment and human. Major reason behind virulent virus vector formation is recombination of the viral vector with wild-type viruses or with related viruses having similar sequences.

(b) *Host range and tissue tropism-associated alterations*:

The viral vector should ideally transfer the genetic material to the target cells with showing any effect on the adjacent cells of healthy tissues (Gogev et al. 2003). Several efforts have been made in direction of developing viral vectors with limited tropism towards the targeted host cell population. As a result, pseudotyped virus vectors are developed, where replacement of viral envelope proteins is done by either proteins encoding envelope from other viruses or by using chimeric proteins. These chimeric vectors will have necessary information for incorporation into virion along with sequence required for interacting specific host cell proteins, for example, the vesicular stomatitis virus glycoprotein (VSV-G) was coated on HIV virus, which is the host range of virus as compared to its parental counterparts which have binding tendency towards CD4 receptors of T helper cells. Reconstruction of viral surface ligands may have several benefits with binding specificity for the target cells, but it comes with the huge concern of mediated side effects, so while developing such pseudotyped viral vectors, there is need of environmental risk assessment (ERA) to ensure that all modifications in the pseudotyped viruses are safe for long-term clinical use and environmental release because these modifications can act in opposite direction by increasing the tropism or the host range pseudotyped vector, which can lead to more severe and novel disease symptoms in humans or animals.

(c) *Changes in susceptibility towards immune system*: A competition has been observed between the host immune system and viruses where host immune responses work in the removal of virus-borne infections from the body by affecting long-term expression and clinical efficacy of viral vectors, whereas viruses evolve mechanisms for invasion of host immune system. This host immune system and virus interaction defines the need of either deleting immune evasion determinants from virus or by encoding immune modulatory function in viral vectors. Attenuation of viral vectors leads to loss immune evasion property, such as E3 deletion from adenovirus or the interleukin (Il)-18 binding protein from poxviruses, which leads to more efficient clearing of the viral vector during an infection and also leads to inflammation as a result of acute immune response

(CHMP 2007). Pathogenic responses may be observed due to addition of genes encoding immune attuning functions which is rare property of wild-type virus. For example, more pathogenic form of poxviruses was formed due to expression of Il-4 which led to inhibition of immune response required for effective clearance of viral infection (Jackson et al. 2001). Enhanced proliferation is one of the possible effects of a viral vector with impaired immune evasion systems in immunosuppressed patients. This aspect should be critically considered during treatment.

9.6 Conclusion

There is no doubt the recombinant biologicals have brought revolution in medical and nonmedical field. They have brought many diseases and other tasks under management by offering new solutions. However, particularly in the field of pharmaceuticals, the complexity of the human body continues to pose new challenges based on the immunological profile of patients. At the core of this are the changes in protein structure that result when they are produced through recombinant DNA technology. While for nonmedical applications, protein's function is the most important parameter, in medical applications, the ability to remain "natural" and evade immune responses is a challenge that persists. Nonetheless, new improvements in protein purification and characterization along with framing of guidelines for quality control of recombinants and the lessons learned during the past years are being put to good use. This will further expand the repertoire of the areas covered by microbial recombinants and offer advantages of new functionalities with cost-effective deployment.

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