Review Article

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Molecular Pharming Of Recombinant Proteins In Plants.

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Short title: Plants Generating Recombinant Proteins.

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ABSTRACT

Plant molecular farming (PMF) refers to modifying the genetic makeup of plants to obtain transgenic plants which in turn are utilised to get recombinant proteins. Recombinant proteins have gained global alertness. Transgenic plants can be utilised to produce various types of recombinant therapeutics. Plants are appropriate and acceptable hosts since the production cost is much less than transgenic animals, fermentation, or bioreactors. Therapeutic proteins such as antibodies, cytokines, enzymes, hormones, and edible vaccines can be produced by incorporating a foreign gene of a desired nature into a suitable plant. Protein production is massive as various types of proteins like antibodies and many others can be produced in molecular farming using transgenic plants. Commercial materials produced through molecular farming attract a big market. Molecular farming opens the opportunity to manufacture affordable modern medicines for global production. Vaccines produced in this field prevent many dreadful viral infections. Commercial sustainability of molecular farming products, proper target selection, purification, modification in production methods, and incorporation of advanced technology are needed. Spectrophotometry and newer techniques like CRISPR/Cas9 have been included in this area of molecular farming. PMF gained much importance during the COVID-19 viral pandemic producing proteins for diagnostic and preventive purposes for making vaccines etc. Viral proteins virus-like particles (VLP) and viral nanoparticles are widely used in recent fields to produce recombinant proteins.

Keywords : Molecular farming, recombinant proteins, non pharmaceutical recombinant proteins, transient expression system.

INTRODUCTION

Molecular farming

Molecular farming is the production of valuable recombinant proteins in transgenic organisms [1]. Transgenic plants are produced by transferring genes from another species into the genome of that plant. Transgenic plants are utilised to express exogenous genes or modify endogenous genes [2]. Genes are responsible for various features expressed in organisms and this is the basis of transgenic technology where external plant genetic features are artificially introduced into the genome of another organism to get desired features. To create a transgenic plant for getting a desired protein, the gene of interest needs to be identified. Such transgenic plants are subjected to molecular farming to produce recombinant proteins., With the advent of new molecular techniques such as CRISPR/Cas9 transgenic plants have unlimited potential. Transgenic plants have increased resistance to various environmental conditions and are more resistant to disease and insects. Herbicide resistance genes generally code for a modified target protein insensitive to the herbicide or for an enzyme that degrades or detoxifies the herbicide in the plant before it can act.

From ancient times plants have been used as sources of medicinal compounds. Transgenic plants are essential for molecular farming, and it has enormously extended the era to produce various specific proteins e.g. plasma proteins, and antibodies whose medical benefits are well understood. Application of molecular biology and plant biotechnology revealed that molecular medicines and vaccines can be produced largely in plants also, forming the basis of molecular farming [3]. Recombinant proteins are complex exogenous proteins. These proteins serve as medical diagnostic reagents and play a vital role in human health care as vaccines, drugs, and monoclonal antibodies. This technology has made a powerful impression on agriculture, basic research, and pharmaceutical industries. In recent years usage of plants

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has significantly increased for the generation of recombinant proteins varying from pharmaceutical therapeutics to nonpharmaceutical products such as antibodies, vaccines, growth factors, cosmetic ingredients, diagnostic reagents, etc Inclusion of methods like mass spectroscopy and gene editing systems in last few years has improved the molecular farming ways. Scientists are also working to produce nutraceutical and food supplements in this field. This is another promising area. Here we tried to review the research articles highlighting different aspects of molecular firming with improvement in outcome as well as its present status.

There has been a lot of enthusiasm for research on various aspects of plant molecular farming since its inception till date. The present authors also find it a very interesting and low-cost method with huge prospects. So, exploring the literature and scrupulous review will further enrich this area of biotechnology through newer innovations and products. This is the novelty of our review.

2. OVERVIEW OF PMF IN THE LAST FEW DECADES

From the initial phase molecular pharming aims to find out newer recombinant proteins for multifarious use particularly those which will help sustain mankind. Gradually plants came as an effective host for such farming. In studying earlier literature, we found that the production of monoclonal antibodies by plant molecular farming in tobacco leaves paved the way to produce medical biomolecules [4]. Monoclonal antibodies serve as vital therapeutic and diagnostic tools in the fields of medicine, health care, and biotechnology [5]. Gradually it was established that polypeptides with desirable characteristics such as smaller-sized molecules, enzyme-fused antibodies, and biological response modifiers can be produced by molecular farming (**Figure 1**) [6] [7].

Figure 1.

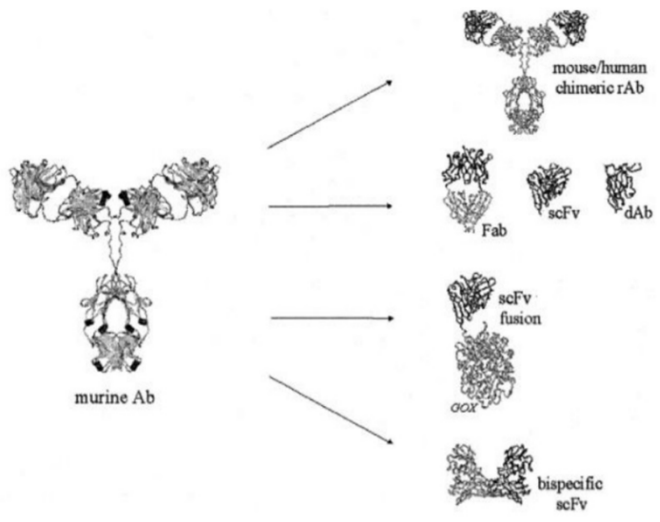

Figure 1. shows various forms of antibodies designed by recombinant engineering [Rainer Fischer et al, Molecular Farming Of Recombinant Antibody In Plants, Institut fur Biologie, 1999].

Besides plants, there are other expression systems like bacterial expression systems or mammalian cell cultures to produce recombinant proteins. But both systems have some drawbacks. The former cannot produce glycosylated full-sized antibodies and contaminated endotoxins are not easily removed. Many times, the recombinant proteins may also turn into inclusion bodies making the process expensive when in vitro folding is essential. Again, Mammalian cell culture is mainly discouraged since it includes costly equipment and media supplements. Use of transgenic animals are legally prohibited {8}. A lot of precautions are taken while working with mammalian cell cultures during downstream processing of recombinant proteins.

Plant-produced recombinant proteins are functional and are almost indistinguishable from their mammalian counterpart. Transgenic plants can also produce organs rich in recombinant proteins for its long-term storage. Transgenic plants can be used as bioreactors for molecular farming of recombinant therapeutic proteins [9]. Molecular farming using transgenic whole plant and plant cell culture systems can produce economically large-scale quantities of antibodies and antibody fragments, antigens and/or vaccines, hormones, and many other biologically active proteins [10]. For fast trans-gene expression in fruits Agrobacterium cultures through fruit stylar apex, resulted in complete fruit infiltration and ultimately this technology was shown to be an efficient tool [11].

Molecular farming produces proteins, but the process does not end there and the methodology of molecular farming needs review from time to time. To get desirable effects with these proteins in various fields they need purification. Purification of proteins is an important area. Also, total yield and quality need to be judged to get a protein for a particular purpose. Affinity chromatography was one method that was used for the purification of therapeutic proteins from transgenic maize using immobilised Histamine [12]. A human antibody was compared against the tumor-associated antigen Tenascin-C (TNC) and found that the yield and quality of plant-produced antibody in either stable transgenics or a transient expression system with promising cost-effective yield [13]. An asexual vegetatively reproductive plant Kalanchoe pinnate was used and found to be a novel and potent bioreactor for plant-based molecular farming using a newly developed transformation method for bio-pharmaceutical proteins [14]. Molecular farming was studied on its various aspects and one such study thought that plant-derived bioproducts show promise of high competitiveness towards classical eucaryotic cell factory systems [15]. Observations also revealed that to produce low-cost recombinant medicines,

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transgenic plants have several advantages over other expression systems and large-scale production is possible. They also can produce complex multimeric proteins with post-transnational modifications [16]. Many of the unique properties of seeds can be exploited in molecular farming applications, particularly where it is desirable to produce large quantities of recombinant proteins. Seeds of transgenic plants have been widely used to generate raw material for the extraction and isolation of proteins and polypeptides, which can be processed into valuable bio-pharmaceuticals [17]. Molecular farming represents an unprecedented opportunity to manufacture affordable modern medicines and to make these available on a global scale. The area of greatest potential is in the prevention of infectious diseases, particularly in underdeveloped countries where access to medicines and vaccines has historically been limited. Production of such medicines and vaccines against HIV, TB, and Rabies was focused and aimed to develop production strategies that are simple and potentially easy to transfer to developing countries [18].

3. REVIEW OF PMF IN THE LAST DECADE

In subsequent years there was the rise in producing commercial products as well as in search for improved technology and market strategy so that products are profitable too keeping scientific research side by side. It was observed that there was a tremendous rise in commercial production of materials using molecular farming throughout the world. DARPA initiative in the U.S.A produced a large-scale influenza vaccine for a successful phase I trial. 'Pharma-Planta academic consortium in Europe gained regulatory approval for a plant-derived monoclonal antibody and completed Phase I human trial. Synth on-the Dutch pharmaceutical company acquired products through their LEX systems based on aquatic plants. Protalix Biotherapeutics- an Israeli biotechnology company received approval for the commercial release of a recombinant form of the enzyme Glucocerebrosidase produced in carrot cells. Now there is global interest in PMF by commercial firms to produce various proteins for multiple purposes. Many companies are now interested in molecular farming and are waiting for more and more products using molecular farming. Commercial sustainability of the products needs a proper selection of appropriate targets This is the most important factor to be considered because of the huge global increase in molecular farming. In the four examples of companies cited above – different molecular technologies were used with different end products and their uses in different areas. In all the above areas molecular farming was a suitable approach, but decision-making in product selection is not an easy job and plant biotechnology is not the only answer for the whole biologic field. In the commercial field desired profit is the aim. So, the current biotechnological approach is that proper beneficial products are targeted in molecular farming from the very beginning [19].

Extraction of proteins from inappropriate sources is associated with problems related to product safety and availability of sources. Molecular farming allows the production of several important recombinant proteins including antibodies, enzymes, pharmaceuticals, etc. Plant molecular farming involves factors like plant hosts, genes of interest, expression vector cassettes, and extraction and purification techniques. Recent advances and strategies of plant molecular farming elements and approaches including promoters, codon optimization, signal sequences, and peptides used for upstream-designed purification and downstream processing need proper discussion. Plant species are a very important factor as a biosynthesis platform in plants to achieve high yields of recombinant proteins. Another important factor is the choice of recombinant gene and its expression strategy. Various studies have been conducted to improve the expression, accumulation, and purification of the recombinant protein from molecular farming systems. In enhancing gene expression at the transcription and translation level reengineered vectors and expression cassettes also play a very important pivotal role. These processes increase protein accumulation and give stability, retention, and targeting of specific organelles [20].

However, some scientists were also concerned about any adverse effects of PMF using transgenic plants. There may be amplification and diffusion of the transgene, toxicity from the accumulation of recombinant protein in the environment, contamination of the food chain, and costs of subsequent processing, etc. so caution to be taken to avoid such complications and keep PMF to produce valuable proteins most cheaply and safely as also in most efficient way [21].

Molecular farming can produce edible vaccines. These vaccines are more acceptable to the community. The status of developed edible vaccines in different plant tissue expression systems needs evaluation regarding the mechanism of action of such vaccines and clinical application with clinical trial stage needs evaluation along with their significance, requirements, advantages, and disadvantages. Edible vaccines have induced both mucosal and systemic immunity. Currently, many pharmaceutical proteins used as edible vaccines have been developed in different plant expression systems and evaluated against dreadful diseases in clinical trials with promising results [22].

Whenever there is some scientific innovation, the question of patenting of the process and the product must be thought of in the interest of the researcher and access to medicines prepared out of recombinant proteins must reach the general population, particularly in the developing world [23].

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4. CURRENT STATUS OF PMF

Molecular farming is an ongoing and promising process using plant-based expression system which offers inexpensive, safe and potentially unlimited way for production of therapeutics in a quick and flexible manner. Experiments for production of various virus like particle (VLP)s and their use in PMF have been established. VLPs are lookalike viruses but do not have infectious genomic materials and they can still elicit a very potent immune response. This makes them ideal vaccine candidate [24]. VLPs can be produced through transient expression systems in plants.

Plant systems have been assessed for the expression of virus like particles of many viruses including polio virus [25], norovirus [26], foot and mouth disease virus [27] and virus like particle [28]. It now has huge progress due to development of newer techniques e.g. CRISPR/Cas9 process. This is a process of plant genetic engineering where gene editing system is incorporated. In recent years the process of gene editing expanded the development of medicines, one of which is production of anti cancer drug from plants. Work in this area showed that these technologies allowed the introduction of plant genetic modification which facilitates the accumulation of native pharmaceutically active substances and even the production of heterologous recombinant proteins. Research revealed that the anticancer agents that are produced by plants naturally or following plant genetic modification specially the proteinaceous anticancer agents can exhibit an improved selectivity and reduced side effects compared to small molecule drugs [29]. Plants are utilized for production of recombinant proteins comprising of both pharmaceutical and non-pharmaceutical proteins (**Figure 2**). Reports clearly suggest the capability of in vivo and in vitro plant systems to develop vaccines for both human and veterinary applications and the antigens produced by plants increase the potential immune responses in model animals and also provides protection in animal challenge experiments. For the development of oral vaccines leafy crops such as lettuces, alfalfa, and clove are used. Also, oral vaccines cut off purification and injections. Booster vaccines obtained from lettuce chloroplast using lyophilized plant cells express polio virus capsid protein which stimulates neutralisation of antibody in mice coupled with inactivated Polio virus and safeguards against all polio stereotypes [30].

Figure 2.

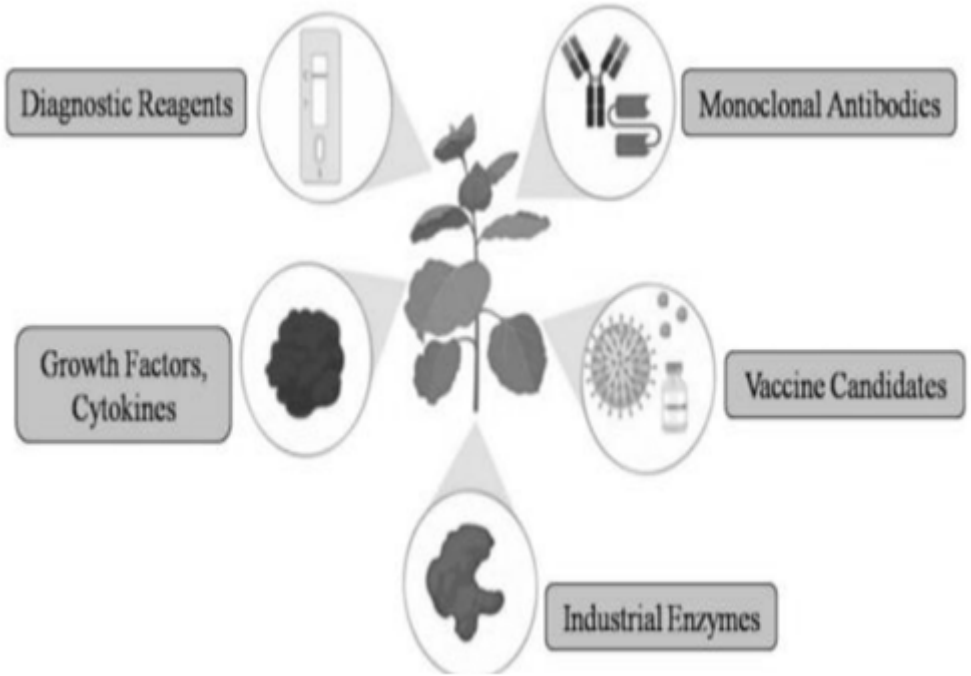

Figure 2. Production of various recombinant proteins through transgenic plants following molecular pharming and it depicts different applications of transgenic plants. [Balamurugan Shanmugaraj et al, Plant Molecular Farming : A viable platform for recombinant pharmaceutical production, 2020].

Moreover, the plant derived virus like particles can serve as reagents and vaccines. Plant based expression system not only play a role in expression of antigens for human diseases but also express antigens for veterinary applications. Non-pharmaceutical protein have been properly analysed for their expression in plant system.

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There is a constant search for improved methods or modifying the available methods of molecular farming to suit a cost-effective production of multifarious products e.g. production of therapeutic monoclonal antibodies (mAbs) whose demands are increasing enormously globally. But these are produced from Chinese hamster ovary cells (CHO) where production and purification cost is very high. A simplified techno economic model for the molecular farming of such antibodies (mAbs) has been indicated recently. Such model for bulk drug manufacturing of monoclonal antibodies (mAbs) can be applied to any production platform. This simplified techno economic model develops economic concepts to identify variables that can be used to achieve cost savings by simultaneously modelling the dynamic costs of upstream production at different scales and the corresponding downstream processing costs for different manufacturing modes --- sequential, serial, and continuous [31].

PMF has extended the production of many biopharmaceuticals e.g. recombinant vaccine antigens, monoclonal antibodies and other commercially viable proteins. In 2020 during the deadly corona virus pandemic importance of already existing molecular farming in producing efficient, cost-effective vaccine in short time was emphasised [32]. Crop based expression systems for instance rice, wheat, corn and legumes are being used recently since they have lesser toxic content as compared to model species like tobacco. Also there exists proper infrastructure for cultivation, harvesting, distribution and processing of crops [33].

In earlier days molecular farming started with limited scope but with years of scientific research it has now gained a very crucial role in protein manufacture. This has become more evident during the dreadful Covid 19 pandemic when millions of human deaths occurred globally. The virus being rather a less known entity, to control the pandemic there came the necessity of rapid production of diagnostic kits, drugs to treat and vaccines to control the future outbreak. Globally scientists undertook significant efforts to design and develop effective vaccines, monoclonal antibodies, immune modulatory proteins, drugs and pharmaceuticals through molecular farming in plants to halt the viral spreads. It is now established that plant molecular farming can rapidly produce biological products in bulk quantity in industrial scale and these products proved to be stable, safe, effective and easily available and affordable. It has potential for fulfilling emergency needs also. In comparison to other major production platforms and technologies plant-based production platforms has emerged as an efficient system for production and that was the need at the time when corona pandemic outbreak started [34]. In Italy in 2020 during the dreadful Covid 19 outbreak molecular farming processes were explored to find out its potential role in rapid and scalable supply of protein antigens as reagents and vaccine candidates, antibodies for virus detection and passive immunotherapy etc. [35]. Molecular farming involves complex biological systems, and some processes were beyond our understanding but studies in the past few years in mass spectrometry-based proteomics has cleared many critical areas of understanding and show promise in advancing our understanding of the complex biological systems. Further, opportunities to leverage comprehensive proteome profiling provided advantage in manufacturing of biopharmaceuticals derived from plants [36]. In covid pandemic huge global demand of vaccines came as an emergency and PMF took the challenge and successfully led to the production of various types of vaccines against the infection. In PMF stable expression in transgenic or transplastomic plants or transient expression was used through viral vectors or agro infection. It is now established that PMF has advantages like low cost, high scalability, safe vaccines with efficient antigenicity and with advantage of being used on membranes like nasal or bronchial mucosa or as oral vaccine [37].

Vaccine production in PMF though an established procedure but newer challenges prevented the process being perfect. Some bioengineering methods were incorporated to get better folding of highly complex proteins and post translational modification. Different cellular engineering techniques or process modifications helped to address these challenges. The cellular engineering process involved glycosylation of plant produced vaccines, gene and construct consideration, modulation of chaperone expression, endogenous oxidase activity, limiting in planta proteolytic degradation, glycosylation of plant produced vaccines, tyrosine O-Sulfation of plant-produced vaccines. Obtaining correctly folded protein is of prime importance in functioning of protein [38].

Molecular farming processes need exploration for production of nutraceuticals and functional food development especially with four major crops – rice, wheat, maize and soyabean. Some scientists showed interest in this area [39]. Malnutrition and undernutrition are still a problem in different parts of globe and functional food and nutracaecals may be an answer.

Molecular farming for production of recombinant proteins for various purposes using transgenic plants has gained huge enthusiasm globally among scientists. PMF has certain advantages over other bioreactors.

Vaccines are used globally to control diseases especially viral. One of the current vaccination challenges include cold chain maintenance, different delivery technologies like injection, micro needle patch etc. To maintain cold chain for delivering vaccines in remote and low resource areas posses' difficulty. Some vaccine needs to be developed for noninfectious diseases e.g. Auto immune diseases and cancer. Often, they need patient specific vaccine which is again a challenge [40].

In PMF plant viruses were used to produce highly efficient expression vector. Various plant viruses are used in PMF

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changing the harmful pathogens into helpful molecular machine and they are useful in producing vaccines against viruses and also are used in bio nanotechnology such as production of virus-based nanoparticles (VNPs). These nano particles are used for vaccine production in plants and there is possibility of using them in oral vaccine treatment also. Plant derived VNPs can be used in various ways in PMF e.g. templates, carriers, containers and scaffold for drug delivery [41].

Figure 3.

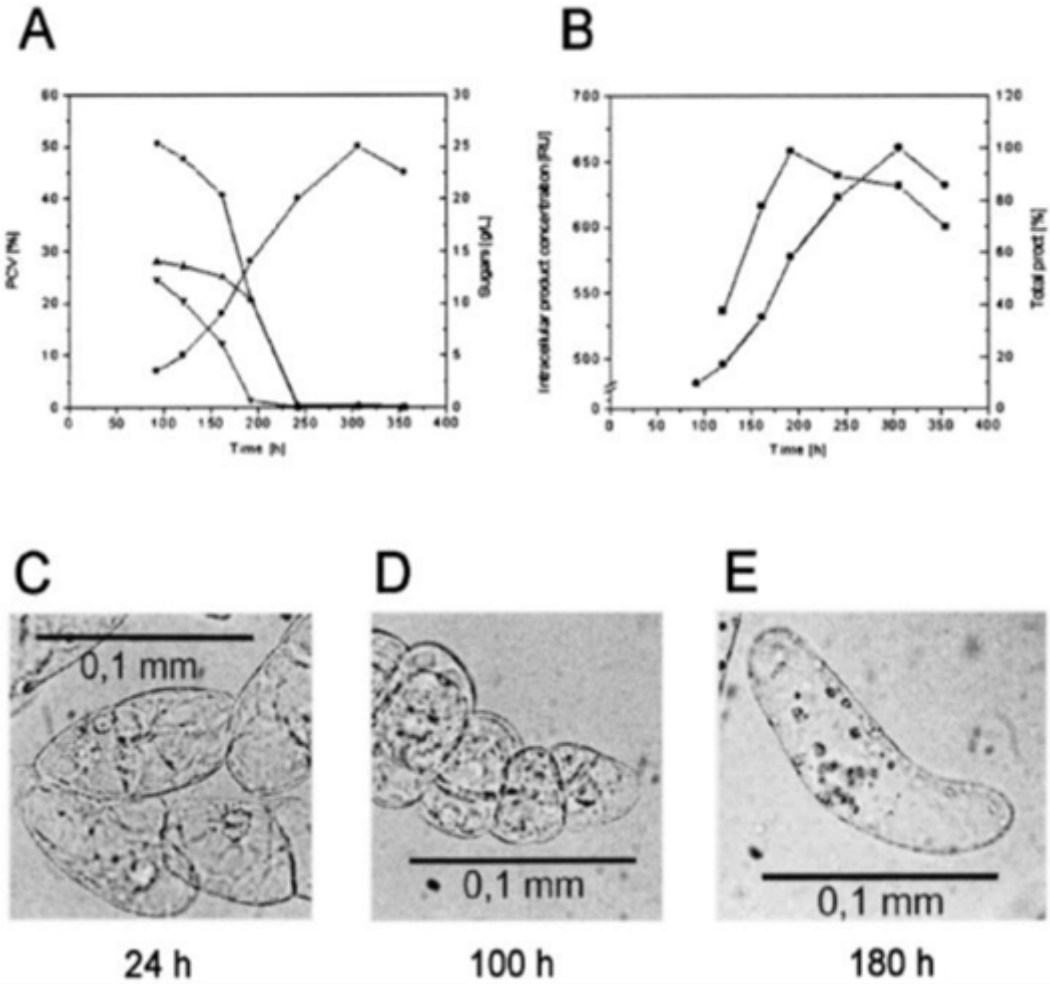

Figure 3. depict production of an Apoplast Targeted Bispecific scFv by fermenting in N. tabacum cv BY-2 suspension cells. Graph (A) indicates uptake of growth and nutrients in a 30-L fermentation of N. tabacum cv BY-2 suspension cells. Sugars present (glucose, fructose, and sucrose) and enzymatically the concentration of sugars in the supernatant was evaluated. (B) cell extract has recombinant bispecific antibody, and it is analyzed through plasmon resonance which is planted on binding of binding of bispecific scFv with one of its antigens. Yield of bispecific scFv is shown in figures C , D and E (per ml culture) . Cells initially show a decrement in size 3 to 5 cell division (24 -100 h) followed by increment (150 - 180 h). [Rainer Fischer et al, Molecular Farming Of Recombinant Antibody In Plants, Institut fur Biologie,1999].

5. DISCUSSION

Various methods are used for molecular farming - Plant Based Expression Systems, Nuclear Transgenic plant system in Terrestrial crops and many others. Nuclear transgenic system is the most extensively used strategy for antibody production in plants. Leafy Crop Systems and Fruit and vegetable crops are also used. Potatoes and tomatoes have been used to produce recombinant antibodies and are much more beneficial than other crops. Potatoes are store organs like seed endosperm so they can store a good quantity of proteins. Antibody production by stable transient expression systems (leafy crops) are used to analyze the working of expression constructs and evaluate the functioning of recombinant proteins before setting up the target for long term transgenic plant production. Agroinfiltration method is an example of transient expression system. Fermentation is another approach adopted for developing recombinant antibodies.

The practice of isolating and purifying the recombinant product is termed as downstream processing and forms the fundamental part of all bio manufacturing processes. Techniques such as affinity chromatography are considered as a standard process for isolation of products.

It is already established that using plant molecular farming and employing plants as bioreactors antibodies, vaccines and medicinal proteins can be produced. Some scientists felt the necessity of production of nutraceuticals and functional food with four major crops rice, wheat, maize and soybean through plant molecular farming. The opportunities and challenges of such functional food production through plant molecular farming in future is worth considering.

The favorable properties of plants as bioreactors for production of recombinant proteins was recognised as early as two decades back and scientific research and review expressed its great prospect [42]. The observation of present scientific research proved it. This is an established technology now and many biotechnological companies adopted this. Now the process is taking up transient expression system which has shown a great potential for rapid production of antibodies in emergencies like pandemic of viral diseases [43]. Critical review of PMF elaborating different methodology including transient expression system, marketing problem, industrial inertia, regulatory hurdles along with its unique protein production to combat emergency like Covid-19 pandemic was undertaken with expectation of broader application of PMF in future. Plants are economically as well as technically more beneficial as compared to conventional system for production of pharmaceutical and non-pharmaceutical products. Plants have the advantage of production of huge quantities of recombinant products at significantly low manufacturing expense.

6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Almost all techniques of Plant Molecular Farming including nuclear, chloroplast expression, transfection of virus etc holds unique attributes which grants them the ability to produce various and different products termed as "targets" within a short duration of time. The result and yield will be enhanced with the fusion of three vital technologies namely, protein engineering, molecular farming and downstream processing. General acceptance of PMF is achieved less due the regulatory framework and the restrictions imposed upon plant extracted products worldwide. The growing demand of industrially and pharmaceutically beneficial recombinant proteins indicates positive future of plant extracted biologics. We hope that in the upcoming days more experiments will be conducted upon transgenic plants.

Studies in the past few years in mass spectrometry-based proteomics has improved molecular farming methods and show promise in advancing our understanding of the complex biological systems. Due to development of newer gene editing techniques like CRISPR/Cas9 process molecular firming will progress further.

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