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Yeast-Based Non-Mammalian Eukaryotic Expression Systems for the Production of Biologics

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Abstract: The study of biologics has grown in importance in the medical field, producing medicines that are vital for the management of numerous disease states. Biologically active substances produced by live cells or through biological processes known as "bioprocessing" are referred to as biologics. They are more complex and therapeutically specific compounds as opposed to tiny molecules that are chemically synthesized. Among other things, biologics include recombinant therapeutic proteins, enzymes, blood products, hormones, vaccinations, and gene and cellular therapies. Prokaryotic and eukaryotic (mammalian and non- mammalian) cells are employed as expression systems for biologic synthesis. Comparing eukaryotic expression systems to prokaryotic-based systems, there are numerous benefits. Using recombinant technology, it has become possible to produce high-quality proteins for clinical application in humans in yeast and filamentous fungal systems. Bioprocessing innovations including genetic engineering and bioreactor design. Improvements in bioprocessing, including genetic engineering, continuous processing, bioreactor design, and quality by design, have made it possible for these non-mammalian eukaryotic systems— which translate proteins similarly to those in mammals—to operate more productively and output more. This article describes the use of eukaryotic expression systems in the production of biologics with significant medicinal potential.

Keywords: biologics; eukaryotic; expression systems; glycosylation; monoclonal antibodies

Introduction: The production of biologics is an industry that is expanding quickly because these specialized medicines provide a targeted approach to treating a wide range of common and chronic illnesses, such as cancer, heart disease, neurological disorders, and autoimmune diseases. The Food and Drug Administration (FDA) defines a biologic as a therapeutic substance that is produced through biological processes using biological systems, as opposed to chemical synthesis (small molecules)[1]. This category of substances includes gene therapies, cell therapy, vaccines, antibody therapies, and non-vaccine therapeutic immunotherapies. In terms of price, manufacture, delivery, and clinical effectiveness, biologics are different from synthetic small molecule medications. Recombinant technology and biotechnology rely on living systems, molecular engineering, and bioreactors (usually submerged state fermentations) to generate big molecules with desired biological activity in order to make therapeutic biologics. Prokaryotic bacterial species, such as Escherichia coli, eukaryotic yeast and fungal systems, such as Saccharomyces cerevisiae, Aspergillus, plant systems, insect systems, mammalian and human expression systems, and cell lines are examples of living systems used as biologic production platforms at an industrial scale.Since small molecules are utilized to treat chronic disorders, they currently make up 90% of global therapeutic sales; nevertheless, the biologics business is growing.The creation of numerous biologically active proteins utilized in disease prevention, treatment, and management has been made possible by recombinant DNA (RDNA) technology [2].The best-selling medication at the moment is Humira (by AbbVie), a recombinant monoclonal antibody (Mab) used to treat autoimmune diseases[3]. Biologics like Herceptin are strong anticancer agents in therapeutic cocktails, they provide treatment choices that are currently unmet[4].The use of chimeric antigen receptor (CAR) T cells in cancer therapy has changed the game. The foundation of CAR T cell therapy is the genetic engineering of patient T cells to target cancer cells that express a particular target antigen[5].Methods for producing biologics devoid of living cells that do not require a cells -cell free system.

Four steps make up bioprocessing: strain/cell line selection and propagation, upstream (fermentation), downstream (processing), and medicine formulation. Typically, a single biologic is created from a single cell strain[6]. Mammalian Chinese hamster ovary (CHO) and murine myeloma cells are the standard bioprocessing systems used to produce many biologics; however, there has been a significant shift towards the utilization of human derived cell lines because post-translational modifications (PTMs) are simple. In many molecular pathways involved in biological activities, PTMs are essential, and PTM mistakes are seen in a variety of disease conditions[7].However, as expression systems for various biologic kinds, fungal and yeast cell systems have significant advantages. Yeast expression systems are durable, inexpensive, feature native PTM machinery, adaptable to genetic engineering or genetic modification (GM), and do not emit endotoxins during processing[8].Yeast are excellent candidates for the production of recombinant proteins because they exhibit both prokaryotic (rapid cell division, single cells, ease of growth) and eukaryotic (cell organelle, PTM activity) characteristics at the same time[9].Characteristics include being pyrogen free, having a high titre value, being inexpensive to produce, and now being categorized as Generally Recognized As Safe (GRAS) organisms[8].Many biotechnology companies use fungal strains, such as Aspergillus and Penicillium, as expression systems to manufacture a variety of biological products, and the FDA considers these strains to be GRAS[10].However, filamentous fungus exhibit intricate morphological traits in submerged cultures that can be difficult to scale up for commercial use, in contrast to unicellular yeast systems[11].

Utilizing Eukaryotic Non-Mammalian Cells in Bioprocessing:

A variety of recombinant proteins can be produced using eukaryotic cell lines, such as CHO cells, human cells, and insect cells[12].However, mammalian cell lines derived from both humans and animals are expensive and vulnerable to microbial contamination, with viral species being the most common cause[13].Recombinant protein technology has advanced, making it possible to express recombinant protein-based biopharmaceuticals in prokaryotic and non-mammalian eukaryotic cells at a lower cost and with greater productivity. This has facilitated the large-scale industrial manufacturing of numerous biologics. Prokaryotic expression systems, in contrast to eukaryotic systems, frequently create proteins that are inert, misfold, produce endotoxins, and are not susceptible to PTM[14].PTM refers to any procedure that modifies the structure of proteins and may involve the addition of a chemical group, such as phosphate, glycosylation of carbohydrates, or ubiquitylation of polypeptides[7].Due to incorrect folding and orientation of the protein within the cell, these changes are frequently associated with the biological activity of the protein, where a loss of functionality may result[15].Since therapeutic glycoproteins make up about 60% of protein biologics, glycosylation in particular is important[16].It is also important to remember that excessive protein glycosylation can have a detrimental effect on enzyme function, including protein stability and enzyme binding[17].PTMs occur in the cytoplasm, endoplasmic reticulum (ER), nucleus, and Golgi apparatus, among other cell organelles[7].Because of this, proteins made using prokaryotic expression systems have to go through an in vitro procedure to insert PTM, which adds stages to the synthesis process and raises costs and decreases yield[16].Furthermore, yeasts are very resilient and tolerant of the challenging fermentation conditions found in bioreactors and large-scale bioprocessing[18].Hence, there are several benefits that non-mammalian eukaryotic systems (such as yeast and fungi) have over prokaryotic systems.

Process of Yeast Cells in the Production of Biologics:

Yeast are unicellular fungus, which are single-celled microorganisms that belong to the Fungus kingdom. Unlike bacteria, which are prokaryotic, yeast are eukaryotic microbial species with cell walls and membrane-bound organelles. Food and drink has long been produced using fungi that develop into yeast morphologies[19].Yeast have long been used as models for the study of mammalian cells, metabolic pathways, and evolution because of their eukaryotic nature. Yeast, when used as host expression systems, can benefit from the same fast growth, high cell density, low cost of medium, and ease of genetic modification that bacteria can offer. Additionally, yeast can perform post-translational modifications, like folding, disulfide bond formation, proteolytic processing, and glycosylation, that are seen in mammalian cells[16].Similar to the PTMs of mammalian cells, yeast frequently undergo alterations such as amidation, hydroxylation, methylation, phosphorylation, pyrrolidone carboxylic acid, ubiquitylation, acetylation, and O-linked glycosylation[20].However, because yeast glycosylation differs from human N- and O-glycosylation, glycosylation still presents a barrier in the creation of recombinant proteins[16].Glycoengineering, enhancing secretory apparatus, and protein breakdown in vivo are attempts to increase glycosylation in yeast and enhance protein folding and stability[8].For this reason, yeast are a great option for the commercial synthesis of recombinant therapeutic proteins. Saccharomyces cerevisiae has long been used to produce recombinant therapeutic proteins. It has been used to produce the Hepatitis B vaccine, insulin, and the hormones glucagon and insulin on an industrial scale[15].In fact, the first recombinant vaccines using S. cerevisiae were developed as a result of the expression of Hepatitis-B surface antigens[21].These species, along with Pichia

pastoris (formerly known as Komagataella phaffii), are being employed as expression platforms for tumor antigens, protozoal proteins, and vaccine production[21].S. cerevisiae also exhibits high osmotic pressure and a wide range of pH tolerance[22]. Three different types of vectors can be used to produce recombinant proteins in S. cerevisiae: centromeric plasmids (YCp), integration plasmids (YIp), and episomal plasmids (YEp)[16].P. pastoris is also receptive to bioengineering, in which strains with altered genetic makeup can create heterologous biologics with glycosylation patterns similar to those of humans[6].Because P. pastoris lacks the ability to make ethanol under aerobic circumstances, it produces a greater yield of protein than S. cerevisiae. permitting increased biomass and protein synthesis[16].The Crabtree effect refers to the fact that S. cerevisiae reduces protein output when it makes ethanol under aerobic circumstances using glucose as a food source, but P. pastoris prefers respiration over fermentation[23].Currently, P. pastoris is used to produce interleukin 1-β, which is used to treat autoimmune diseases, interferon-α, which is used to treat cancer, hepatitis B and C, and macrophage colony-stimulating factor (M-CSF), which is used to treat hematological disorders[23].Among other things, P. pastoris is used to produce human insulin, human serum albumin, the hepatitis B vaccination, trypsin, and collagen[16].Unlike the well-established conventional S. cerevisiae, P. pastoris is still a non-traditional yeast whose genome has not yet been fully identified, making genetic engineering more difficult[16].Fully active heterologous proteins, such as pochymosin, which S. cerevisiae secretes little, may be produced and secreted by P. pastoris with great efficiency[24].The capacity of yeast to produce protein products provides several benefits for the synthesis of biologics, such as simpler separation and purification processes and the absence of harmful intracellular heterologous protein accumulation, which lowers production costs[18]. The capacity of the unconventional yeast Yarrowia lipolytica to create heterologous recombinant proteins is well-known. Unlike S. cerevisiae, Y. lipolytica is a human commensal, obligate aerobe, GRAS that can fold and secrete large and/or complicated heterologous proteins[25].In fact, this species is less likely to experience hyperglycosylation, a characteristic of recombinant S. cerevisiae proteins[26].Many heterologous valuable metabolites, such as carotenoids, terpenes, polyketides, molecules derived from aromatic amino acids, and therapeutic biologics such as interferon α, epidermal growth factor, blood coagulation factor XIIIa, proinsulin and insulinotropin, cytochrome P450 enzymes, and oestrogen receptor α, are produced by engineered strains of Y. lipolytica [27]. Even though Y. lipolytica has a similar yield and productivity to S. cerevisiae and P. pastoris, only 25 of the approximately 150 recombinant proteins that have been generated thus far are produced at the industrial or bioreactor scale[28].Challenges with the strain's oxygen requirements, dimorphism, and metabolic burden prevent scale-up[28].The unconventional yeast Ogataea polymorpha, originally known as Hansenula polymorpha, has been employed as an expression system to produce insulin-like growth factors and hepatitis B vaccinations[29].Because H. polymorpha can withstand temperatures between 30 and 50 °C, it may produce proteins that are biologically active at 37 °C. This is similar to what has been observed in other yeast, such as S. cerevisiae, which has decreased hyperglycosylation and PTM[30].

Non-Ribosomal Peptides and Polyketides:

A class of tiny molecules known as polyketides and non-ribosomal peptides, with intricate chemical structures, are created by plants, marine creatures, and microbial species. These compounds facilitate self-defense, communication across species, and environmental adaptation [21]. The enzymes polyketide synthases (PKSs) and non-ribosomal peptide

synthetases (NRPSs) are responsible for the biosynthesis of polyketides and non-ribosomal peptides, respectively [31]. Numerous compounds or their hybrids have anticancer, immunosuppressive, and antibacterial properties, such as vancomycin, rapamycin, and calecheamicin, making them clinically significant as biologic therapies [32]. The use of yeast in enzymatic biochemical processes for the synthesis of these biologics has gained traction recently. For instance, the filamentous fungus Aspergillus terreus, which produces the anti-cholesterol medication lovastatin, is usually the source of yeast S[17].

Production of Vaccines

The manufacture of vaccines has recently shifted toward protein-based, virus-like particle, viral vector, and nucleic acid-based approaches in an effort to address the problems with pathogenicity, immunogenicity, biocompatibility, and time restrictions associated with classic vaccine types [8]. Subunit-based and viral-like particle-based vaccines are produced using recombinant technology and microbial expression methods [33]. Indeed, whole yeast-based vaccines (WYVs) and yeast platforms have emerged for the treatment of cancer and the fight against infectious diseases [8]. Strong adjuvant qualities, long-term antigen stability, ease of GM, and the capacity to survive the gastrointestinal system enabling oral delivery are just a few of the numerous benefits that the non-pathogenic S. cerevisiae provides in the vaccine-making process [34]. Regardless of yeast cell viability, investigations have demonstrated that entire recombinant S. cerevisiae cells expressing foreign antigens may activate DCs, strong antigen-specific cytotoxic T lymphocyte (CTL) responses, and impart protective cell-mediated immunity in animal tests [35]. Yeast-based vectors (WYVs) that express certain tumor antigens have the potential to be used in immunotherapy to treat cancer, including melanoma, papilloma, leukemia, and carcinoma [24].

Monoclonal Antibodies:

Glycosylation of monoclonal antibodies (mAb) is essential to their biological activity and is used in the treatment of infectious diseases, inflammation, and cancer [36]. Because incompatible surface glycosylation prevents most antibody molecules from being produced in yeast expression systems, only a small number of antibody molecules have been functionally expressed in yeast systems [18], with CHOs cells continuing to be the primary source of mAb synthesis on an industrial scale. The interaction between the antibodies and immune system effector cells as well as biological activity are impacted by the glycosylation of mAbs at the Fc region [36]. Humans have an immunogenic response as a result of S. cerevisiae-induced hyper-mannose glycosylation of mAbs [8]. P. pastoris is less likely to experience this hyperglycosylation of mannose [16]. Targeted genetic engineering has been used in experimental experiments to enhance the glycosylation of mAb generated in yeast [36]. CRISPR-cas9 technology has enabled genetic modification of individual genes, gene families, or the whole genome [37]. However, the limited tumor penetration, high production costs, immunogenicity, and potential for treatment resistance of mAbs impede their use as biologics [38]. There are certain benefits to therapy based on polyclonal antibodies (pAbs), which are a mixture of synergistic mAb acting on many epitopes. pAbs like ZMapp, which combines three mAb to treat Ebola, and the anticancer pAb combination of rituximab and lumiliximab have enhanced antitumor activity [6].Antigen-binding fragment (Fab), single-chain variable fragment (scFv), and single V-type domain are examples of biologics referred to as minimal antibody-binding fragments. These fragments have several benefits, including increased tissue penetrability, stability, solubility, decreased immunogenicity, and less expensive industrial scale production [18]. Large-scale specific antibody fragments, or recombinant antibody

fragments (rAb), may be created using RDNA technology and microbial expression systems. These antibody fragments are simpler to extract than those made in mammalian cell culture systems. It has been possible to express rAbs using yeast S. cerevisiae and P. pastoris, as well as prokaryotes E. coli and Bacillus subtillis [39].Obstacles such manufacturing time and ideal cryopreservation procedures need to be looked into in order to get the high yield needed for therapeutic application [39]. The use of antibody fragments as therapeutic biologics is restricted due to their brief serum half-life and immunogenicity caused by aggregation [40]. In engineered yeast surface display (YSD) technologies, antibodies fragments are frequently employed through a genetic fusion process between the antibody fragment and the yeast cell surface antibody fragment [40]. For the treatment of Hodgkins lymphoma, the PD-1 blocking antibody Sintilimab, which was produced by yeast display technology, was approved [41].The most recent antibody-driven research, especially in the detection and treatment of cancer, has been prompted by the finding of heavy-chain only antibodies (HcAbs), also known as nanobodies, in camelids [38]. Because of their durability, minimal immunogenicity, and capacity to exhibit a variety of anti-tumour targets, nanobodies are a viable option for therapeutic usage [38]. Research reports on the utilization of a modified S. cerevisiae as a platform for in vitro nanobody production [42].

Industrial Aspects to Take into Account:

Building and genetically modifying the strain for bioreactor scale-up is the initial stage in the synthesis of yeast recombinant proteins [9]. Using plasmids or expression vectors that transfer the desired gene to the host cells, the GM of host cells for the synthesis of heterologous proteins entails the overexpression of some host genes as well as the partial deletion of other host genes [26]. Heterologous DNA can be incorporated into the host chromosomal DNA or it can be episomally maintained as a plasmid; the latter is more common in yeast species [26]. Unlike prokaryotes, which lack introns, eukaryotic genetic material is composed of protein-coding sections called exons that are encircled by non-coding areas called introns [43]. Splicing introns to produce mRNA is the process of identifying the intriguing gene (one that codes for a biologically active protein). An expression cassette with an open reading frame including the protein gene for expression regulation and a promoter is required for heterologous synthesis in yeast cells [26]. Although it's not necessary, a terminator region can be inserted to stop transcription. The terminator region stops transcription, affects protein gene expression levels, and strengthens mRNA stability, all of which increase protein production [29].An expression cassette's transcription may also be greatly increased by the insertion of an intron [28]. Growth hormone is one of the many human genes that require introns for appropriate expression, as many other genes also greatly increase the transcriptional output of these genes [44]. A promoter is a brief DNA region that serves as the beginning point for RNA polymerase to transcribe a gene. Constitutive and inducible promoters are the two types of promoters found in yeast. Well-known constitutive and inducible promoters with potent transcriptional activity are used in bioprocessing to increase the required biologics' overproduction in yeasts [16,45].Inducible promoters are typically used when growth and production must be separated, whereas constitutive promoters enable simplicity and stable expression levels [16]. There is some control over the amount of gene expression since induced promoters can change their transcriptional activity in response to stimuli like as carbon sources and environmental conditions [45]. Chemically promoting protein expression or altering the bioreactor's fermentation environment minimizes metabolic burden and lessens the toxicity of recombinant proteins to host cells [46]. Methanol may be used as a carbon source by methylotrophic yeast, such as P. pastoris and H. polymorpha, which enables the use of methanol-inducible promoters [30].When inducible promoters are

used in fed-batch bioreactors at an industrial scale, cells can grow quickly before protein expression occurs, which can have some impact on metabolic load [28]. Researchers elsewhere have characterized the most often used yeast promoters [16]. It is highly desired to create synthetic promoters that enable higher protein production, improved protein folding, and better controlled transcription [28]. Three nucleotides that are necessary for protein translation are known as codons, and codons that encode the same amino acid are referred to as synonymous codons. These codons are subject to species-specific use bias [47].Research has indicated that codon use, which controls translation elongation and translational protein folding processes, is a significant factor in determining mRNA stability in Saccharomyces cerevisiae [48]. Research is necessary to ascertain how codon optimization affects protein production in yeast expression systems [49]. Conventional yeast like S. cerevisiae is encouraged to be used in the design and bioengineering of recombinant yeast due to the genetic tractability and usage of genetic tools. Understanding the genetic makeup, metabolic processes, and biochemical systems of non-conventional yeast is essential for optimizing the use of strains like Y. lipolytica in bioprocessing, where their advantages over S. cerevisiae may be capitalized upon. For instance, Y. lipolytica, which is regarded as a dimorphic yeast, possesses genomic features in common with filamentous fungus, according to genome sequencing [26]. The morphological condition of Y. lipolytica in bioreactors, where the variability of dimphorism affects heat and mass transmission in the reactor and consequently affects protein production, has not been fully understood [28]. For the manufacture of recombinant proteins, further non-conventional yeasts include P. pastoris, K. lactis, Y. lipolytica, H. polymorpha, and S. pombe [9].

Cell type, medium composition, substrate concentration, biocatalyst cell density, product inhibition, pH, and temperature all have an impact on how well bioreactors operate, and continuous mode yields higher productivity than batch systems [50]. Yeast cells may produce more recombinant proteins by optimizing the medium composition, temperature, pH, cell growth, and protein synthesis kinetics. Crucially, a lack of certain amino acids and insufficient energy might cause translational mistakes in the recombinant protein, which can affect the immunogenicity and stability of the protein [28]. Because they are foreign proteins present in the cell, heterologous proteins synthesized in the host cell may be susceptible to proteolytic destruction [46].One way around this restriction is to genetically modify yeast strains that lack protease [9]. While keeping cells inside the bioreactor helps to save expenses while simultaneously increasing cell density, increasing cell density also boosts product yield. When setting up a bioreactor, dissolved oxygen (DO) is a crucial factor to take into account since it affects the kinetics of aerobic cell growth, cell physiology, and stability. During the exponential phase, when cells are proliferating and metabolically active, DO needs increase [28]. Fed-batch and continuous reactor cell densities are complicated by procedures that are susceptible to the Crabtree effect, need a variety of equipment types for setup, have high operating costs, require lengthy times for downstream operations, and require extensive cultivating times [51]. Cell retention in a continuous system can be achieved by using cellular flocculation, immobilization on a carrier material, or membrane filtering of the product [50]. When a heterologous protein is expressed, the host cell's metabolism is sacrificed in order to focus cell resources on the recombinant protein's transcription and translation. This allocation of resources to heterogeneous protein creation at the expense of cellular activity, decreased growth capacity, and specific growth rate is referred to as metabolic load [28]. Moreover, higher recombinant gene sizes, copy numbers, expression levels, and problems with dissolved oxygen and nutrition availability all increase the impact of metabolic burden [52].The extraction and purification steps of downstream processing are significantly streamlined when

the target protein product is secreted [26]. In response to signals that have a direct bearing on the yield of protein, the process of transporting the protein across the membrane of the endoplasmic reticulum (ER) initiates protein secretion [9]. Redox enzymes and protein folding chaperones should be overexpressed in yeast systems because misfolded protein is broken down before release and can harm the ER [9].Research outlines the simultaneous synthesis of many medications or drug combinations in a single batch, which has a number of benefits over a single biological production [6]. Recently, the topic of synthetic biology has gained popularity in the medical and therapeutic sciences with applications in the development of medications, vaccines, and biosensors [53]. Synthetic biology is the integration of engineering into bioprocessing technologies and the construction of customized systems intended for a biologic goal. Because of their many benefits, E. Coli and S. cerevisiae are the most often used expression systems for synthetic biology [54]. By manipulating gene circuits that affect protein levels, expression levels, and pathway levels of expression systems through promoter engineering, synthetic biology enables increased biologic production.

In conclusion

Yeast are categorized as either non-methylotroph or methylotroph species for the purpose of producing biologics. P. pastoris, a methylotrophic yeast, Hansenula polymorpha, and S. cerevisiae, a non-methylotrophic yeast, are used in the industrial setting to produce recombinant proteins. Yeasts that are beneficial expression systems include Kluyveromyces lactis, Y. lipolytica, and Komagataella sp. S. cerevisiae is a desirable production system for a range of biologics because it is a non-mammalian eukaryotic expression system, has good expression levels, is devoid of toxins, and can execute PTMs. Because PTMs affect protein folding, which causes misfolded proteins to lose stability and function, they are related to the biological activity of proteins. Despite the fact that yeast glycosylation differs from that of mammalian cells, the application of RDNA technology has made it possible to create genetically modified strains of yeast that somewhat mimic human glycosylation. Additionally, S. cerevisiae has certain significant drawbacks, including as poor secretion of proteins larger than 30 kDa, regulatory promoter deficiencies, and unsuitability for high- density culture. These restrictions have prompted the use of unconventional yeasts, such as P. pastoris, as expression systems.Filamentous fungi as expression platforms for biologics have several benefits, such as high density growth flexibility, high rate of protein production with low medium needs, glycosylation and other PTMs more akin to human proteins. In fact, it is said that filamentous fungi like Aspergillus and Trichoderma have 10 times more secretory capacity than S. cerevisiae. Because non-mammalian eukaryotic cells consume less energy and produce less waste than mammalian systems, they are also used in more ecologically friendly industrial production methods. A novel approach to vaccine delivery is provided by the use of yeast expression systems to create recombinant protein, virus-like particles, and yeast surface display for development as oral vaccines. Improving eurkaryotic manufacturing platforms might lead to higher drug production productivity soon. Because biologics are more expensive than small molecule therapy, many patients cannot afford them and hence do not choose biologics for treatment. Lower manufacturing costs may make these powerful medicines more widely available, facilitating greater access and more advanced medical treatment on a worldwide scale.

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