# ANHUI POLYTECHNIC UNIVERSITY

## College of Biology and Food Engineering

# Construction and Expression Optimization of Recombinant Human Lactoferrin in *Pichia pastoris*

重组人乳铁蛋白毕赤酵母工程菌的构建及表达条件优化

Student name: MERYEM DAHMANI

Supervisor: Dr. **ZHENGLIAN XUE**

Specialty: molecular biology

Research direction: Development and Application of Industrial Microbial Resources

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**Abstract**

Lactoferrin a multifunctional glycoprotein. It is used in the culinary, pharmaceutical and nutraceutical sectors. It is particularly useful in medicine and conservation thanks to its recognized antibacterial, immunomodulatory and iron-binding properties. There is an urgent need to develop more efficient recombinant production systems, as conventional milk extraction techniques are still constrained by low yields (typically 0.2-1.5 g/L) and exorbitant production costs.

1. In this work, we used the *Pichia pastoris* (*P. pastoris*) to create an optimal expression platform. The pPIC9K vector was successfully used to clone the human lactoferrin gene (NCBI: NM_002343.2), which was then introduced into *P. pastoris* GS115 host cells. Further molecular analysis by SDS-PAGE and colony PCR electrophoresis confirmed the stability of 80 kDa recombinant protein integration and expression. With inhibitory zones measuring 12.3 0.5 mm against Staphylococcus aureus, 8.7 0.3 mm against *Escherichia coli* and 14.1 0.6 mm against GS115 yeast, functional validation showed high bioactivity and confirmed preservation of the protein's natural antimicrobial properties.

2. Through an orthogonal $L16(4^3)$ experimental design and extensive optimization, we were able to determine the most important factors for maximum production efficiency. 1% (v/v) methanol induction, 0.1 g/L iron supplementation of $Fe^{3+}$ and a well-balanced medium formulation with soy and beef extract (10 g/L -20 g/L) as nitrogen source and corn syrup (1 g/L) as carbon source were ideal conditions. Exceptional yields of 640 mg/L were achieved under these ideal bottle agitation conditions, an increase of two times compared to baseline production levels.

3. The scale-up fermentation of the 5L bioreactor verified the scalability and robustness of the process. The fermentation kinetics showed that under the conditions of controlling dissolved oxygen, pH and methanol supplementation every 24 hours, the cell density reached OD600 up to 58 at 36 hours, and then it was basically stable. The lactoferrin yield reached a peak of 693.94 mg/L at 48 hours after induction.

This study showed that *P. pastoris* can be used as a platform for the production of human lactoferrin with high expression and biological activity. Compared with the traditional extraction method, it has significant advantages in terms of production efficiency and economic benefits.

Keywords: Lactoferrin ; *Pichia pastoris* ; Recombinant protein ; Optimization of expression conditions

**摘要**

乳铁蛋白是一种多功能糖蛋白，广泛应用于食品、医药和营养保健品领域。其公认的抗菌、免疫调节和铁结合特性使其在医疗和防腐方面具有特殊价值。由于传统乳源提取法存在产量低（通常为 0.2-1.5 g/L）、生产成本高昂等问题，开发高效重组生产系统迫在眉睫。

1.本研究利用毕赤酵母（*Pichia pastoris*）构建了优化表达平台。通过 pPIC9K 载体成功克隆了人乳铁蛋白基因（NCBI: NM_002343.2），并转化至毕赤酵母 GS115 宿主细胞中。经 SDS-PAGE 电泳和菌落 PCR 分子鉴定，证实了 80 kDa 重组蛋白的稳定整合与表达。功能验证显示其具有优异的生物活性：对金黄色葡萄球菌的抑菌圈为 12.3±0.5 mm，大肠杆菌为 8.7±0.3 mm，酵母 GS115 为 14.1±0.6 mm，表明该蛋白天然抗菌特性得以保留。

2.通过单因素和 $L16(4^3)$正交试验设计和系统优化，确定了影响生产效率的关键参数最佳条件为 1%（v/v）甲醇诱导、0.1 g/L $Fe^{3+}$铁离子补充，以及以玉米糖浆（1 g/L）为碳源、豆粕-牛肉浸膏（10 g/L -20 g/L）为氮源的培养基配方, 在此优化摇瓶条件下，产量达到 640 mg/L，比优化前提升 2 倍。

3. 5L 生物反应器发酵放大验证了该工艺的可放大性和鲁棒性。发酵动力学显示, 在控制溶氧、pH、每 24 小时补充甲醇条件下，36 小时细胞密度即 $OD_{600}$可达 58，此后基本稳定。诱导 48 小时乳铁蛋白产量达峰值 693.94 mg/L。

本研究表明毕赤酵母可作为兼具高表达量和生物活性的人乳铁蛋白生产平台。相比传统提取法在生产效率和经济效益方面具有显著优势。

**关键词：**乳铁蛋白; 毕赤酵母; 重组蛋白; 表达条件优化

# List of figures

# List of tables

# 目录

# Chapter 1: Introduction

## 1.1 Overview of Lactoferrin

Lactoferrin (LF) is a multifunctional glycoprotein belonging to the transferrin family. The term "lacto" refers to its origin from milk, while "ferrin" denotes its iron-binding capacity. Its name arises from its ability to bind iron, imparting a distinctive red color. Lactoferrin is composed of approximately 700 amino acids, with a molecular weight of about 80 kDa and an isoelectric point of approximately 8.0 (Ebrahim, M., et al., 2014). It is found in substantial amounts in various body fluids, including milk, saliva, urine, and tears. In cow's milk, lactoferrin is naturally present at an average concentration of about 0.2 g/L. In human milk, the concentration ranges from 2-4 g/L, while in colostrum, it is even higher, ranging from 6-8 g/L. Bovine colostrum contains approximately 1.5 g/L. Lactoferrin, a member of the transferrin family, is primarily recognized for its roles in immunological defense and iron transport. It is a highly adaptable glycoprotein that binds iron. The first milk produced after childbirth, colostrum, contains notably high levels of lactoferrin (Ebrahim, M., et al., 2014). As colostrum is the first immune-boosting material provided to infants, the presence of lactoferrin is crucial for early immunological protection. Furthermore, lactoferrin is stored in neutrophil granules and released at infection or inflammatory sites, underscoring its role as a key component of the innate immune system's first line of defense against infections (Jiang, Z., et al., 2008).

Lactoferrin's extensive biological activities, including antibacterial, anti-inflammatory, antioxidant, and immune-regulating properties, have attracted considerable attention for its potential therapeutic applications. Its unique ability to limit the availability of iron—a critical resource for many pathogens—makes it a natural inhibitor of microbial growth, positioning it as a promising agent for immunological health and infection treatment (Kongsinkaew, M., et al., 2023). As a result, lactoferrin is widely investigated for its role in disease prevention and immune support, and it is utilized in a variety of nutritional and pharmaceutical products (Iglesias-Figueroa, B., et al., 2016).

However, the lactoferrin obtained through the separation and purification of milk does not meet the growing market demand. Therefore, there is an urgent need for the development of novel technologies for lactoferrin production. One such approach involves the use of

microorganisms to produce human lactoferrin through recombinant DNA technology. The development of synthetic biological systems has provided the feasibility of using microorganisms to generate large quantities of lactoferrin. Lactoferrin from various species has been successfully expressed in both prokaryotic and eukaryotic systems, as well as other expression systems. In this review, we will describe the design and construction of lactoferrin expression systems, highlight some of their applications in the field of synthetic biology, and discuss the associated challenges and opportunities (Shimazaki, T., 2000).

### 1.1.1 Structure and Physicochemical Properties of Lactoferrin

Lactoferrin (Lf) is a multifunctional glycoprotein from the transferrin family, noted for its high-affinity binding to iron ($Fe^{3+}$). Lactoferrin consists of around 700 amino acids and has a molecular weight of approximately 80 kDa. The protein has a bilobed configuration, with N-terminal (N1, N2) and C-terminal (C1, C2) lobes, interconnected by an α-helix. The lobes exhibit significant homology, with roughly 40% sequence identity, and each lobe is also separated into two domains. The fissure between these domains functions as the iron-binding site, necessitating the presence of an anion such as carbonate for coordination (Baker, H., & Baker, S., 2009).

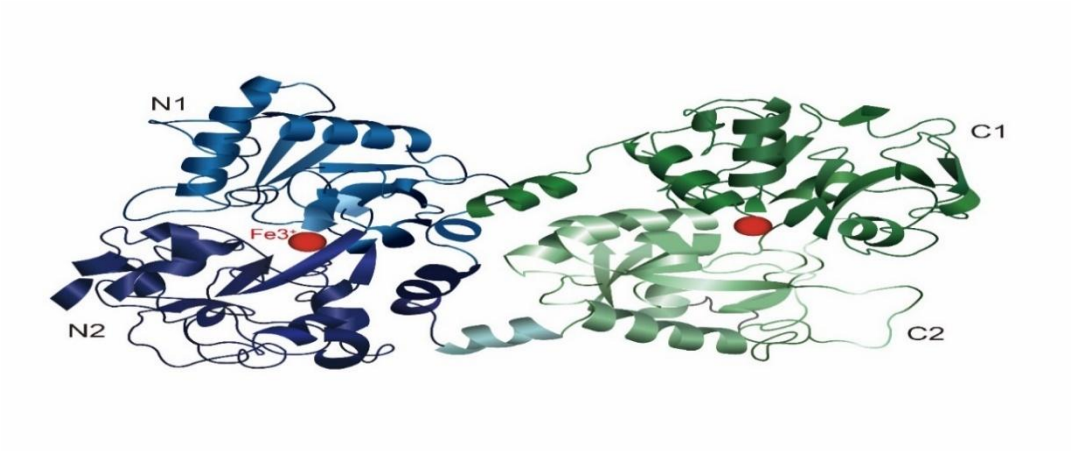



**Fig. 1-1 Lactoferrin structure**

Illustrates the lactoferrin structure, with the N-terminal (N1, N2) and C-terminal (C1, C2) lobes clearly visible. The binding of iron ($Fe^{3+}$) in the cleft of the lobes of the glycoprotein lactoferrin is usually what gives it its biological action.

The secondary structure of lactoferrin demonstrates its functional plasticity and structural

stability, both of which are crucial for its biological functions. Predicted structural studies indicate that alpha helices comprise roughly 42.5% of the protein's secondary structure. Extended strands constitute 20.8%, producing stable beta-sheet sections that enhance the protein's structural integrity. Beta turns, constituting 12.7%, are essential for linking structural motifs, whereas random coils, making up 24.0%, provide the necessary flexibility for dynamic interactions with ligands and receptors. This distribution underscores lactoferrin's capacity to preserve structural integrity while enabling many biological tasks, including iron binding and antibacterial action (Baker, H., & Baker, S., 2009). (Table 1-1)

The secondary and tertiary structures of lactoferrin are reinforced by many disulfide connections, rendering it highly resistant to proteolytic destruction. Moreover, the structure of lactoferrin is affected by its glycosylation, featuring asparagine-linked oligosaccharide chains at certain locations. In humans, two of the four possible N-linked glycosylation sites are occupied, but in bovine lactoferrin, three out of five sites are glycosylated. The physicochemical features of lactoferrin are intricately associated with its biological functions. Its capacity to sequester iron underlies its antibacterial and immunomodulatory functions by depriving microorganisms of this vital mineral. The protein has a high isoelectric point, enabling interaction with negatively charged surfaces like bacterial membranes, therefore demonstrating bacteriostatic and bactericidal properties (Iglesias-Figueroa, B., et al., 2016).

Structural analyses have demonstrated notable similarities between human and bovine lactoferrin, with a sequence identity of around 69%. Notwithstanding some variations in the relative orientation of their lobes and domain closure, their three-dimensional structures are mostly maintained. The presence of lactoferrin sequences from other species, such as rats, horses, pigs, and camels, in the Protein Data Bank (PDB) has facilitated comparative analysis of their structural and metabolic characteristics (Choi, S., et al., 2008).

The advancement of recombinant lactoferrin has enabled its functional and structural analysis. Recombinant lactoferrin, produced in systems such as P. pastoris, maintains its bioactivity, encompassing antibacterial and iron-binding characteristics. Progress in recombinant technology has underscored the significance of post-translational modifications, including glycosylation, in defining its physicochemical and functional characteristics (Iglesias-Figueroa, B., et al., 2016).

Table 1-1Predicted secondary structure of lactoferrin

| Structure | Percentage (%) |
|---|---|
| Alpha helix (Hh) | 42.5 % |
| Extended strand (Ee) | 20.8% |
| Beta turn (Tt) | 12.7% |
| Random coil (Cc) | 24.4 % |

### 1.1.2 Physiological Functions of Lactoferrin

Lactoferrin (Lf) is a multifunctional glycoprotein with diverse biological roles, primarily involved in iron regulation, immune modulation, antimicrobial defense, and antioxidant activity. The ability of lactoferrin to transition between its iron-bound (holo-lactoferrin) and iron-free (apo-lactoferrin) states makes it highly adaptable to various physiological processes. This adaptability enables lactoferrin to perform multiple functions that are critical for maintaining health.

Lactoferrin plays a pivotal role in regulating iron homeostasis in the body. It binds to iron tightly, preventing its free availability, which can otherwise lead to oxidative damage. In its holo-lactoferrin form (iron-bound), lactoferrin serves as an iron transporter, facilitating the delivery of iron to cells and tissues. In contrast, apo-lactoferrin (iron-free) is involved in iron sequestration, limiting the iron available to pathogens and preventing iron-induced oxidative stress (Choi, S., et al., 2008).

The dual functional states of lactoferrin—holo-lactoferrin and apo-lactoferrin—allow it to adapt to the body's varying iron requirements. This makes lactoferrin essential for managing iron levels in the body while also protecting against iron overload and deficiency (Iglesias-Figueroa, B., et al., 2016).

Lactoferrin is well-known for its potent antimicrobial activity. It exhibits broad-spectrum activity against bacteria, fungi, and viruses by interacting with their cell walls and membranes. In the case of bacteria, lactoferrin binds to lipopolysaccharides (LPS) on the outer membrane of Gram-negative bacteria, causing membrane disruption. It also interferes with the cell surface features of Gram-positive bacteria, preventing microbial adhesion and invasion. This antibacterial action is enhanced by lactoferrin’s ability to sequester free iron, which is essential for bacterial growth (Jo, W.,

et al., 2011).

Moreover, lactoferrin's antiviral properties are equally important. It can bind viral particles or host cell receptors, thereby preventing the virus from entering host cells and inhibiting replication. This mechanism is critical in protecting against a variety of viral infections, showcasing lactoferrin as an effective agent in viral disease prevention (Kongsinkaew, T., et al., 2023).

In addition to its antimicrobial functions, lactoferrin plays a key role in regulating inflammation. It modulates the immune system by interacting with various immune cells, including macrophages and neutrophils. Lactoferrin helps regulate the production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). By inhibiting these cytokines, lactoferrin prevents excessive inflammation that could lead to tissue damage or chronic inflammatory conditions (Choi, S., et al., 2008).

Additionally, lactoferrin promotes the release of anti-inflammatory cytokines, ensuring that the immune response is balanced and does not become overactive. This modulation of inflammation is essential for protecting tissues from damage while maintaining the body’s ability to respond effectively to infections (Jo, W., et al., 2011).

Lactoferrin also plays a vital role in boosting the immune system. It enhances the function of various immune cells, including macrophages, neutrophils, natural killer (NK) cells, and lymphocytes. For example, lactoferrin increases the phagocytic activity of macrophages, allowing them to better engulf and eliminate pathogens. It also stimulates the activity of NK cells, which are responsible for identifying and destroying infected or abnormal cells, such as tumor cells (Ebrahim, M., et al., 2014).

By influencing both innate and adaptive immune responses, lactoferrin helps maintain immune homeostasis, enhancing the body’s ability to defend against infections and combat abnormal cells (Iglesias-Figueroa, B., et al., 2016).

Lactoferrin has been shown to possess antioxidant activity, which is crucial for protecting cells from oxidative damage. It reduces the formation of reactive oxygen species (ROS) by binding to free iron, which otherwise contributes to ROS production. Oxidative stress is linked to aging and a variety of inflammatory diseases, and by mitigating this stress, lactoferrin helps protect tissues from damage (Jiang, Z., et al., 2008).

This antioxidant effect extends to cancer prevention as well. Oxidative stress plays a significant

role in tumorigenesis, and lactoferrin's ability to reduce oxidative damage may contribute to its potential as an anticancer agent. Recent studies suggest that lactoferrin may prevent tumor growth by inducing apoptosis in cancer cells and inhibiting angiogenesis, the process by which tumors develop new blood vessels to support their growth (Kongsinkaew, T., et al., 2023).

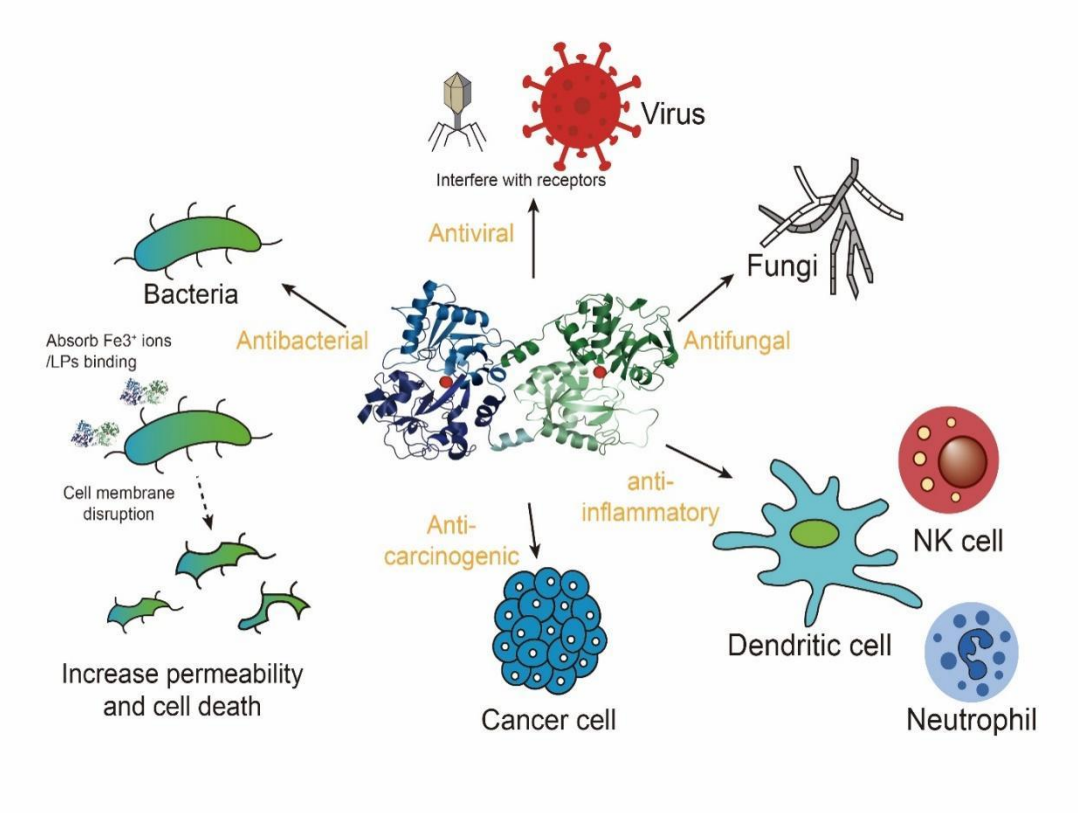



Fig. 1- 2: Multifunctional roles of lactoferrin

### 1.1.3 Applications of Lactoferrin

Lactoferrin (Lf) has numerous beneficial biological activities, leading to its wide applications in medicine, nutrition, and healthcare. These applications are based on its roles in iron regulation, immune modulation, infection prevention, and antioxidant protection.

Lactoferrin is commonly used in neonatal care, particularly in infant formulas, to mimic the protective functions of human breast milk. It supports gut maturation, fosters a healthy gut microbiota, and provides antimicrobial defense, reducing the risk of conditions like necrotizing enterocolitis (NEC) in preterm infants (Jiang et al., 2008). Lactoferrin also helps treat iron-deficiency anemia by improving iron absorption without the side effects of conventional iron supplements, benefiting those with high iron needs, such as pregnant women and infants (Hao et al., 2019).

In oncology, lactoferrin shows anticancer properties by inhibiting tumor growth, inducing apoptosis in cancer cells, and modulating immune responses to boost tumor immunity. Preclinical studies have suggested that it may help suppress tumor growth in the liver, colon, and stomach (Choi et al., 2008). Moreover, lactoferrin is used in the nutraceutical industry as an immune booster, antimicrobial agent, and food preservative, addressing consumer demand for natural, clean-label products (Yang et al., 2018).

Recombinant lactoferrin is effective against various infections due to its antimicrobial properties. It exhibits antibacterial activity by sequestering iron, disrupting bacterial membranes, and preventing biofilm formation, working against both Gram-positive and Gram-negative bacteria (Legrand et al., 2005). Lactoferrin also inhibits viral replication, including for viruses like HIV, influenza, and herpes simplex (Andersen et al., 2006), and demonstrates antifungal activity against fungi such as *Candida albicans* (Acosta-Zaldívar et al., 2017). Additionally, it shows antiparasitic activity, inhibiting the growth and survival of malaria and *Trypanosoma* parasites (El-Ansary et al., 2018).

Lactoferrin holds promise in cancer therapy due to its anti-cancer properties, including inhibition of tumor cell proliferation, apoptosis induction, and modulation of immune responses. It has been shown to inhibit tumor growth and metastasis in several cancer types, including breast, colon, prostate, and lung cancers (Tsuda et al., 1996). Lactoferrin also has antiangiogenic properties, preventing the formation of new blood vessels that tumors require for growth (Lin et al., 2007). Additionally, lactoferrin boosts immune cell activity and protects healthy tissues from radiation damage during cancer treatments (Meng et al., 2013).

## 1.2 Production Methods of Lactoferrin

Lactoferrin is conventionally isolated from bovine milk using methods like ion-exchange chromatography and ultrafiltration. Nonetheless, these techniques can be expensive and may produce restricted outputs (Fee & Chand, 2006). Recombinant DNA technology has been utilized to generate lactoferrin in many systems, such as transgenic animals and plants, to tackle these issues. Recombinant human lactoferrin has been effectively produced in the milk of transgenic cows, offering a feasible large-scale manufacturing approach (Nuijens et al., 1997; Platenburg et al., 1994).

### 1.2.1 Extraction-Based Production of Lactoferrin

Lactoferrin (LF) is a valuable iron-binding glycoprotein found in bovine milk and whey, widely

used in the food, pharmaceutical, and nutraceutical industries (Fee & Chand, 2006). Over time, researchers have developed various methods to extract LF, each with its own strengths and limitations. One of the most common techniques is ion-exchange chromatography, which takes advantage of LF's natural positive charge at neutral pH. This allows it to bind to negatively charged resins like CM-Sephadex C-50 (Sharbafi et al., 2011). While this method is effective in producing high-purity LF, it comes with high costs, complex multi-step processing, and a delicate balance between yield and purity (Fee & Chand, 2006; Yoshida & Ye, 1991; Nuijens et al., 1996).

Membrane-based separation methods, such as ultrafiltration and microfiltration, rely on size exclusion to isolate LF. These techniques are relatively simple but lack selectivity, often requiring additional purification steps. Moreover, membrane fouling is a common problem that reduces efficiency and increases operating costs (Pawar et al., 2019; Sharbafi et al., 2011). Another emerging method is reverse micellar extraction (RME), which uses surfactant-based systems like CTAB/n-heptanol to separate LF from whey. While this approach has shown high extraction efficiency, it still requires careful optimization and precise surfactant selection to be viable for large-scale use (Platenburg et al., 1994; Nuijens et al., 1996).

Batch extraction with cellulose phosphate has also been explored, particularly for human whey, achieving an impressive 96% purity and 80% yield. However, its scalability for bovine whey remains uncertain (Yoshida & Ye, 1991; Sharbafi et al., 2011). Despite these advancements, current extraction methods remain costly and often difficult to scale up efficiently. As demand for lactoferrin continues to grow, researchers are working toward more economical and practical large-scale production solutions that can make LF more accessible while maintaining its functional properties.

### 1.2.2 Biosynthetic Production of Lactoferrin

Recombinant DNA technology has emerged as a useful substitute for conventional extraction techniques in the synthesis of lactoferrin biosynthesis, helping to overcome the problems of scalability, high price, and low yield (Choi et al., 2008). Transgenic animals, such as cattle, sheep, and rabbits, are an established method. These animals are designed to produce recombinant human lactoferrin (rhLF) in their milk (Nuijens et al., 1997). The protein is most similar to human lactoferrin due to the high yield and natural post-translational modifications made possible by this process. But it is a complicated solution due to ethical issues, long development period, and stringent regulatory requirements (Platenburg et al., 1994). Plant-based expression systems, which genetically modify

plants such as maize and tobacco to manufacture lactoferrin, are another potential strategy (Wang et al., 2008). Lower expression levels and variations in post-translational modifications provide difficulties for plant-derived lactoferrin, despite its cheapness, lack of animal-derived pollutants, and scalability (Yang et al., 2018). High expression capacity and rapid manufacturing are provided by microbial expression methods, which include yeasts such as *P. pastoris* and bacteria such as E. coli (Andersen et al., 2006). While bacterial systems are cheap and simple to work with, yeast-based systems allow glycosylation, which increases protein stability. However, bacterial expression can lead to problems such as improper protein folding, lack of post-translational modification, and potential endotoxin contamination (Acosta-Zaldívar et al., 2017)

#### 1.2.2.1 Recombinant Lactoferrin Expression Systems

Compared to traditional expression systems such as E. coli and mammalian cells, P. pastoris offers a balance of cost-efficiency and post-translational modification capability. While E. coli grows rapidly and is inexpensive, it cannot perform glycosylation, often resulting in misfolded or inactive proteins. Mammalian cells ensure proper folding and modification but are expensive and slow-growing. In contrast, P. pastoris combines high cell density growth, ease of genetic manipulation, and the ability to glycosylate proteins, making it a versatile system for expressing complex eukaryotic proteins like lactoferrin.

With the growing demand for lactoferrin (LF) in medicine, nutrition, and biotechnology, scientists have developed recombinant production methods to overcome the limitations of traditional extraction from milk. Several biological systems have been explored for this purpose, including bacteria, fungi, transgenic animals, and mammalian cell cultures. Each system has its strengths and challenges, but yeast pacifically *P. pastoris* has gained significant attention due to its efficiency, scalability, and cost-effectiveness.

Bacterial systems, particularly *Escherichia coli*, are widely used for producing recombinant proteins due to their rapid growth and high yield. However, bacteria struggle to express complex glycoproteins like lactoferrin because they lack the cellular machinery for proper glycosylation. As a result, the protein often misfolds and forms inactive aggregates called inclusion bodies, requiring complex refolding steps that reduce efficiency and increase costs (Yuan et al., 2019).

Fungal expression systems, such as *Aspergillus niger*, offer an alternative approach. These

systems can secrete proteins directly into the culture medium, simplifying purification. However, fungi produce a range of unwanted proteases that can degrade the target protein, and their glycosylation patterns differ from those in humans, which may impact the biological activity of recombinant lactoferrin (Wang et al., 2020).

Transgenic animals, such as cows and goats engineered to produce lactoferrin in their milk, provide a natural environment for protein expression with proper post-translational modifications. While this method can yield large amounts of high-quality lactoferrin, it involves ethical concerns, long breeding cycles, and strict regulatory barriers, making it difficult to scale for commercial use (Zhang et al., 2008).

Mammalian cell cultures, like Chinese hamster ovary (CHO) cells, are another option. These cells closely mimic human protein production, ensuring correct folding and glycosylation. However, mammalian cell cultures are expensive to maintain, require complex bioreactor systems, and produce lower yields compared to other systems, limiting their feasibility for large-scale lactoferrin production (Zhou et al., 2018).

Among these expression systems, *P. pastoris* has emerged as one of the most effective platforms for lactoferrin production. This yeast combines the rapid growth and high-yield advantages of bacterial systems with the ability to perform eukaryotic post-translational modifications. *P. pastoris* secretes the target protein directly into the culture medium, simplifying downstream processing and reducing costs (Yang et al., 2019). It also features a strong methanol-inducible promoter system (AOX1), which allows for tightly regulated, high-level protein expression under controlled conditions. Unlike mammalian cells, *P. pastoris* is cost-effective, grows to high densities, and is easier to scale up for industrial production (Zhao et al., 2021).

Overall, while various recombinant systems exist, *P. pastoris* stands out as the most promising option for large-scale lactoferrin production. Its ability to balance cost, efficiency, and protein quality makes it a leading choice for industrial applications in pharmaceuticals, nutrition, and biotechnology.

#### 1.2.2.2 Optimization of Recombinant Lactoferrin Expression Parameters

The optimization of lactoferrin in *P. pastoris* include a number of intricate and methodical procedures. The gene for human lactoferrin (hLF) is first produced and optimized for expression in the *P. pastoris* host. The gene is subsequently cloned into an appropriate expression vector and

introduced into P. pastoris cells by electroporation methods to generate recombinant strains. Positive clones are evaluated for their capacity to express lactoferrin, and the most effective construct is chosen for subsequent scale-up investigations. Microscale culture is performed to ascertain the ideal conditions for protein expression. The procedure is further optimized by high-throughput screening and productivity assessment to improve yield. Lab-scale fermentation is conducted under regulated settings to examine expression parameters and refine medium composition, temperature, pH, and induction duration. Extensive fermentation systems are subsequently employed to generate lactoferrin in commercial quantities. The expressed lactoferrin is purified, characterized, and its biological functions are confirmed to confirm that its quality and activity satisfy the required criteria. This systematic approach guarantees the effective manufacture and scalability of recombinant lactoferrin with elevated purity and functionality.

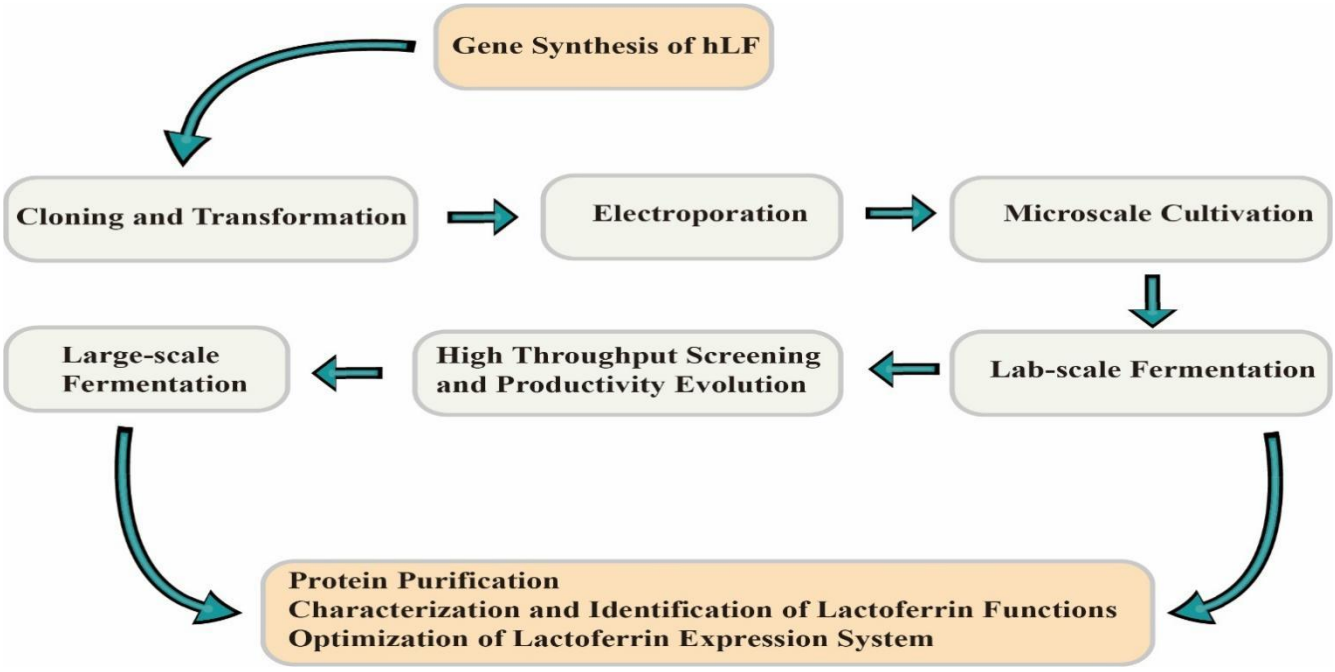



**Fig. 1- 3: Producing and optimizing lactoferrin in *P. pastoris* technical process**

1.3 Separation and Purification of Lactoferrin

A variety of separation techniques have been developed in accordance with the characteristics of lactoferrin (Table 1-2) A high-concentration salt solution can compete with lactoferrin for water molecules, destroy the water film on the surface of protein colloidal particles, and cause protein precipitation. (Wong, 2018. Luo et al,2010) extracted lactoferrin from bovine colostrum by salt-out

and organic reagent precipitation from bovine colostrum. They achieved a 48% recovery of lactoferrin after salt-out with 80% $(NH4)_2SO_4$ at pH 8.5 and precipitation with 60% ethanol.The extraction technique did not necessitate costly experimental equipment, and the operational procedure was straightforward. However, the extracted lactoferrin had a low purity.

The utilization of chromatographic techniques, such as ion-exchange chromatography, gel filtration chromatography, and affinity chromatography, is commonly employed for the purification and separation of lactoferrin, as depicted in Figure 3 by (Moradian et al.,2014) These methods rely on the positive charge, size, or shape of the protein for separation and purification. Big Beads is a strong cation exchanger with granules of 200 m. (Fan et al.,2017) used a 0.05 M sodium phosphate buffer solution to equilibrate the Sorbent SP Sepharose Big Beads to extract lactoferrin from raw milk. The recovery rate achieved by this method was exceeding 90%, with a protein purity of 95% Lactoferrin chromatographic separation often has a long cycle time and requires excessive pressure when droplets pass through the chromatographic column. It is also difficult to distinguish impurity proteins with the same charge and similar isoelectric point, which limits the purity of obtained lactoferrin and leads to high cost (Fan et al., 2017).

A magnetic nanoparticle can be controlled by a magnetic field. When an electric field is applied, the magnetic nanoparticle immediately assumes magnetic properties and is capable of swimming under the actuation of the electric field. When the applied electric field is removed, the magnetism immediately disappears, which is conducive to the separation of magnetic nanoparticles. Using magnetic nanoparticles and membrane technology, lactoferrin can be quickly separated from milk. Chen, L. et al. (2007) synthesized micrometer-sized monodisperse superparamagnetic polyglycol methacrylate particles coupled with heparin through polymerization and then purification. Lactoferrin was recovered directly from acidic whey using magnetic PGMA-heparin particles using a one-step method using NaCl solution as the eluent. The maximum lactoferrin binding capacity was 164 mg/g, which was higher than the commercial standard for protein purity.

Due to the wide application of lactoferrin, the requirements for the purity of lactoferrin have increased due to the widespread application. The development of new methods could greatly improve the purity of lactoferrin. Wu and Xu (2009) used chromatography and membrane-binding methods to separate lactoferrin and immunoglobulin G at the same time. The purity was 95.0% and 96.6%, respectively. However, recovery rates were relatively low, only 68.83% and 45.38%, respectively,

(Wu & Xu, 2009) Lu et al. (2007) isolated lactoferrin from bovine colostrum by ultrafiltration, and then purified it using a fast-flowing strong cation exchange chromatography system. The optimal operating parameters for ultrafiltration were set at a tangential flow rate of 4 m/s, a transmembrane pressure of 150 kPa, and an operating temperature of 50°C. The purity of the concentrated lactoferrin reached 30.88% (w/w) and the recovery rate was 94.04%. The crude lactoferrin was purified by preparative strong cation exchange chromatography. According to (Lu et al.,2007), the final lactoferrin product exhibited a purity of 94.20% and recovery of 82.46.

Table 1-2 Extraction separation and purification method of lactoferrin

| Extraction and separation type | Method | Effect recovery | Advantage or disadvantage | References |
|---|---|---|---|---|
| Cation exchange chromatography | XK16 | Protein adsorption capacity reached 48.6 mg/ml | Simple operation and few steps | Feeand Chand (2006) |
| Membrane adsorption | Cation exchange membrane | Lactoferrin purity 94% | Maintain protein activity | Wolman et al. (2007) |
| Colloidal gas | Anionic surfactant sodium | Lactoferrin recovery rate 90 | Continuous operation large | Fuda et al. (2004) |

## 1.4 Research Significance and Content

### 1.4.1 Research Significance

Lactoferrin, a multifunctional glycoprotein recognized for its iron-binding capacity and its antibacterial, antiviral, and immunomodulatory activities, has garnered considerable interest for its therapeutic potential in healthcare and industry. Its many biological roles render it a crucial element in nutritional supplements, medicines, and cosmetic uses. The capacity for recombinant lactoferrin manufacture has facilitated large-scale manufacturing to satisfy increasing worldwide demand.

This work involved the construction of a high-yield recombinant human lactoferrin (rhLF) expression strain of *P. pastoris* utilizing modern molecular biology techniques, such as gene optimization and codon adaptation. The optimal conditions for boosting P. pastoris expression levels were established by high-throughput screening and fermentation optimization. Multiple parameters affecting expression efficiency and yield were carefully examined to provide an optimum production platform for lactoferrin.

1.4.2 Research Content

In this work, a recombinant human lactoferrin (rhLF) strain will created in Pichia pastoris. Expression conditions will optimize and fermentation will scale up in a 5 L bioreactor. Critical actions included:

**1.Construction of human lactoferrin**

For the genetic construction, condoning the human lactoferrin gene (NCBI NM_002343.2) into the pPIC9K vector using standard restriction enzyme digestion and ligation techniques. The recombinant plasmid will then be transformed into Pichia pastoris GS115 competent cells through electroporation. Following transformation, plan to plate the cells on YPD agar containing increasing concentrations of G418 antibiotic to select for multicopy integrands. Potential positive clones will be verified through colony PCR screening and DNA sequencing of the integrated expression cassette.

**2. Process Optimization Phase**

Conduct systematic optimization in shake flasks to determine ideal expression conditions. This will involve: Testing methanol induction at different concentrations (0.5% and 1.0% v/v) with daily supplementation. Evaluating iron supplementation levels (0.05 g/L vs 0.1 g/L FeCl3) while controlling Ph. Performing medium component screening using an L16 orthogonal array design to assess various carbon sources, nitrogen sources, and metal ion combinations

**3. Bioreactor Scale-Up Phase**

For production scale-up, will implement a controlled fed-batch fermentation process in a 5L bioreactor. The strategy will consist of: Initial glycerol batch phase for biomass accumulation.Methanol induction phase with continuous feeding of optimized methanol .Throughout the fermentation, monitor and control key parameters including temperature, pH, dissolved oxygen, and agitation speed, taking regular samples for analysis.

# Chapter 2: Materials and Methods

## 2.1 Experimental Materials

The strain used for recombinant expression was P. pastoris GS115 (Strain No. 5), which was preserved in our laboratory and selected based on prior screening for optimal protein expression capacity.

### 2.1.1 Plasmids, Primers, and Strains

*E. coli* DH5α, *P. pastoris*, Yeast GS115, *Staphylococcus aureus* expression vector pPIC9K were all preserved in the laboratory.

Table 2- 1: Strains and plasmids

| Strains/ Plasmids | Descriptions | Sources |
|---|---|---|
| *E. coli* DH5α | Strains | TaKaRdalian |
| *P. Pastoris* | Competent cells | Ourlab |
| *S. aureus* | Carrier carrying lactoferrin Bacteriostatic test verifies bacteria | Our lab |
| Plasmids | | |
| PpIC9K-hLF | Plasmids are constructed in this paper | Our lab |

### 2.1.2 Main Experimental Reagents

The restriction endonucleases, Primestar max, Speedone buffer, and Loading buffer used were from Thermo Fisher Scientific; DNA Marker and forward and reverse primers were synthesized from Shanghai Bioengineering Co., Ltd.; protein marker was purchased from TaKaRa; gel recovery kit was purchased from OMEGA was purchased from Guangzhou Feiyang Bioengineering Co., Ltd.; Geneticin (G418) and Coomassie Brilliant Blue Fast Staining Solution were purchased from Beijing Solebow Technology Co., Ltd.; Plasmid extraction kit was purchased from Guangzhou Meiji Biotechnology Co., Ltd.

### 2.1.3 Key Instruments and Equipment

A PCR thermal cycler was used for DNA amplification, while a gel electrophoresis system analyzed PCR products and plasmids. DNA and protein concentrations were measured using a

spectrophotometer. A high-speed refrigerated centrifuge facilitated cell pelleting and DNA purification. An electroporator introduced recombinant plasmids into *P. pastoris*. Incubators and shakers ensured optimal culture growth, and an autoclave sterilized media and tools. SDS-PAGE and Western blot apparatus verified lactoferrin expression.

2.1.4 Common Solutions and Culture Media

Table 2-2 Culture Media

| Culture medium name | Instrument model | pH requirement |
|---|---|---|
| LB Solid Medium | Peptone 10, yeast powder 5, NaCl 10, agar 2% | Natural pH |
| LB Liquid Medium | Peptone 10, yeast powder 5, NaCl 10 | Natural pH |
| YPD Solid Medium | Yeast powder 10, peptone 20, glucose 20, agar 2% | Natural pH |
| YPD Liquid Medium | Yeast powder 10, peptone 20, glucose 20 | Natural pH |
| GPD Solid Medium | Yeast powder 10, peptone 20, glycerol 20, agar 2% | Natural pH |
| GPD Liquid Medium | Yeast powder 10, peptone 20, glycerol 20 | Natural pH |
| BMMY Medium | Yeast extract 10, peptone 20, dissolved in 700 mL distilled water, sterilized by high-pressure steam at 121°C for 20 min, add 100 mL 0.1 M phosphate buffer, 100 mL 10x methanol, 100 mL 10x YNB, 2 mL 200x Biotin | N/A |

2.1.5 Databases and Major Software Tools

The NCBI database or related literature reports (NM-002343.2), GenScript Biotech Co., Ltd., restriction enzymes EcoRI and HindIII, PCR and gel electrophoresis, sequencing software

2.2 Experimental Methods

2.2.1 Primer Design and Synthesis

The hLF gene from the NCBI database or related literature reports (NM-002343.2), synthesized at GenScript Biotech Co., Ltd., ensuring high specificity and efficiency for PCR amplification. The primers were then validated and optimized for successful amplification of the target gene.

2.2.2 PCR Amplification of Target Gene

Once the primers were synthesized, the amplification of hLF gene using Polymerase Chain Reaction (PCR). The reaction included the synthetic primers, Primestar max DNA polymerase, dNTPs, and template DNA. Verify the PCR product by agarose gel electrophoresis and recover it.

2.2.3 Construction of Recombinant Expression Vector

Find the hLF gene from the NCBI database or related literature reports (NM-002343.2), synthesized at GenScript Biotech Co., Ltd., amplified with the synthetic gene as a template, obtained the recombinant plasmid by seamless cloning technology and the plasmid PpIC9K with shore resistance through EcoRI and HindIII double cutter, introduced it into DH5α, and then introduced into *P. pastoris* competent state by electroporation, and coated on a plate containing ampicillin. Pick a single colony and inoculate it into liquid culture medium containing resistance. After cultivation, extract the plasmid and use it as a template for amplification. Verify the PCR product by agarose gel electrophoresis and recover it. After sequencing, it shows that the constructed plasmid is correct. The strains and plasmids involved in this study are shown in Table 2-1.

2.2.4 Preparation of Competent *E. coli*

*E. coli DH5α* was made competent by a treatment with calcium chloride (CaCl ) to improve the absorption of DNA. The recombinant plasmid pPIC9K-hLF was introduced into E. coli by thermal shock transformation, after selection on LB agar plates containing ampicillin. Individual colonies were collected, cultured in liquid LB medium and the plasmids were extracted using a plasmid extraction kit. The extracted plasmid was verified by restriction digestion and sequencing before processing by *P. pastoris*.

2.2.5 Preparation and Transformation of *P. pastoris* Competent Cells

To produce the recombinant hLF protein. The validated plasmid pPIC9K-hLF was electroporated on *P. pastoris* GS115. The yeast cells were first made competent by treatment with lithium acetate, dithiothreitol (DTT) and sorbitol, which increased membrane permeability. The plasmid was then introduced using an electroporator, and the transformed cells were spread on geanticline-containing YPD agar plates (G418) to select positive transformants., Recombinant yeast strains were cultured for

protein expression analysis after PCR screening of positive colonies. This transformation process allowed *P. pastoris* to integrate and express the hLF gene in a stable manner, which enabled the production of recombinant lactoferrin for purification and characterization.

### 2.2.6 Colony PCR Verification of *P. pastoris*

Colonies of *P. pastoris* were analyzed after transformation to confirm the successful integration of recombinant plasmid pPIC9K-hLF. This analysis was performed immediately by PCR on the colonies of certain transformants. Individual colonies were selected from YPD agar plates containing genticine (G418) and suspended in sterile water. The cells were then heated to lyse, releasing genomic DNA used as a PCR matrix.

A high-fidelity DNA polymerase was used to ensure process accuracy, combined with specific direct and reverse primers designed to amplify the hLF gene. Agarose gel electrophoresis was used to analyze the PCR results, and the presence of an appropriately sized DNA band suggested successful plasmid integration. In order to ensure the successful generation of recombinant strains of *P. pastoris*, colonies with positive amplification were selected for further validation and protein expression studies.

### 2.2.7 Protein Quantification Analysis

To evaluate lactoferrin expression, modified strains of *P. pastoris* were grown under breeding conditions. G418 (geneticine) has been added to the medium to ensure the survival of successfully transformed bacteria. Two different assays of G418 (500 mg/mL and 3 200 mg/mL) were used as controls to monitor selection effectiveness. After activation, the strains were seeded to antibiotic-containing YPD agar and specific colonies were selected for further examination. Before being injected into agitated vials for large-scale development, these colonies were grown in YPD liquid medium for a full day.

### 2.2.8 Qualitative Identification of Lactoferrin

The lactoferrin engineered bacteria were activated, and G418 (Geneticin) wasadded to the culture medium as a screening mechanism during activation. Concentrations of 500 mg/ml and 3200 mg/ml G418 were used as parallel controls for the cultured bacteria. After activation, they were

streaked onto YPD solid culture medium plates containing antibiotics, and single colonies were picked and cultured in YPD liquid test tubes for one day before being inoculated into shake flasks for culture. After 24 hours, 0.5% pure methanol was added to each shake flask to induce expression, and SDS-PAGE electrophoresis was used to determine whether the engineered bacteria expressed lactoferrin.

2.2.9 Antimicrobial Activity of Lactoferrin

This experiment uses the double-layer plate method for antibacterial testing. Take about 10 ml of the completely dissolved LB and YPD solid culture medium, cool it to 60°C, and evenly pour it into the culture dish as the first layer of culture medium. Cool and solidify it in the clean bench. After solidification, place an Oxford cup in the bottom culture medium. At the same time, place the remaining LB and YPD in a 50°C water bath to prevent solidification. Mix the bacterial solution diluted to a reasonable OD600 with the corresponding culture medium and pour it into the plate, and mark the Oxford cup serial number on the plate. Add 80 μL of fermentation supernatant to the Staphylococcus aureus plate with Gram-positive bacteria. ①-③The fermentation supernatant was 1-3 days old. On the plate of Escherichia coli, the strain was Gram-negative bacteria.①Add 40 μL of sterile water from the second day's fermentation supernatant into the Oxford cup.②Add 80 μL of methanol to the Oxford cup to induce the fermentation supernatant on the second day.③Add 120 μL of methanol to the Oxford cup to induce the fermentation supernatant of the next day.①Add 100 μL of methanol to the Oxford cup to induce the fermentation supernatant of the first day.②Add 100 μL of methanol to the Oxford cup to induce the fermentation supernatant on the second day.③Add 100 μL of methanol to the Oxford cup to induce the fermentation supernatant on the third day.④Add 100 μL of methanol to the Oxford cup to induce the fermentation supernatant on the fourth day. Seal with plastic film and place the culture dishes containing Escherichia coli and Staphylococcus aureus in a 37 ℃ incubator, and the culture dishes containing *P. pastoris* in a 30 ℃ incubator. Observe the results after 48 hours of cultivation.

2.2.10 Optimization of Induction Conditions for Lactoferrin Expression

To maximize lactoferrin expression in *P. pastoris*, a series of experiments were conducted to optimize key induction conditions, including methanol concentration,and iron ion concentration. in this study was precisely quantified using the Coomassie Brilliant Blue method, a well-established

and dependable methodology for protein measurement. This technique depends on the interaction of Coomassie Brilliant Blue dye with proteins, leading to a colorimetric shift that can be quantitatively assessed. The binding largely transpires via non-covalent interactions between the dye and the protein's basic and aromatic amino acid residues, resulting in a change in the dye's absorption spectrum.

An ultraviolet spectrophotometer was utilized to quantify the absorbance of the protein-dye combination at a wavelength of OD595.

This wavelength is ideal for measuring the color intensity of the complex, establishing a direct relationship with the protein content in the sample. The utilization of a spectrophotometer facilitated accurate and consistent measurements, minimizing variability and guaranteeing data dependability.

A critical stage in this procedure was the formulation of a standard curve utilizing known amounts of bovine serum albumin (BSA) as a reference protein. The standard curve was created by graphing the absorbance readings at OD595 in relation to the relevant BSA concentrations. This curve functioned as a calibration instrument, facilitating the determination of protein concentrations in the experimental samples. The precise protein concentration in the fermentation supernatant was evaluated by comparing the absorbance of the experimental samples to the standard curve.

The standard curve was meticulously developed to provide a spectrum of values, guaranteeing it spanned the anticipated protein levels in the samples. This thorough method reduced mistakes and improved the precision of the quantification. The Coomassie Brilliant Blue technique, in conjunction with a UV spectrophotometer and a meticulously prepared standard curve, established a solid and dependable framework for quantifying protein concentration. The precise measurement method was essential for evaluating the efficiency of lactoferrin synthesis and the success of the optimization strategies employed in this investigation.

We conducted a concentration gradient experiment comparing 0.5% and 1.0% (v/v) of methanol in agitated flask cultures to ascertain the optimal methanol concentration for lactoferrin induction. We conducted a concentration gradient experiment comparing 0,05 g/L and 0.1 g/L of FeCl3 in agitated flask cultures to ascertain the optimal Fe3+concentration for lactoferrin induction. To thoroughly evaluate the variables affecting lactoferrin synthesis, a methodical approach was employed to screen various sources of carbon, nitrogen, and metals in shake flask fermentation

medium. These components were chosen based on their availability, price, and potential to enhance the production of recombinant proteins. The selection procedure was carefully planned as a three-factor, four-stage orthogonal experiment, providing a solid basis for studying the influence of these elements, separately or in combination, the ability of *P. apastoris* to produce lactoferrin.

Glycerol, glucose, corn syrup and water were the sources of carbon analyzed; they represented different metabolic processes and energy production. To ensure consistency, each source of carbon was evaluated at a fixed concentration of 1 g/Based on preliminary tests, this dose was sufficient to support protein synthesis and development without affecting metabolism. Nitrogen sources, necessary for amino acid synthesis and cellular metabolism, were evaluated at concentrations ranging from 10 to 20 g/L. These included peptone, beef extract, soybean meal powder and yeast extract. The optimal range to stimulate lactoferrin synthesis while reducing waste of resources and potential metabolic disturbances was determined using different nitrogen concentrations. Metals were evaluated simultaneously as essential cofactors influencing protein stability and enzyme activity. The metals evaluated were FeCl3, ZnCl2, MnCl2 and CuCl at 0.1 g/L, all concentrations remained constant. This concentration was chosen precisely to ensure sufficient availability for cellular functions while avoiding dangerous levels.

The experimental technique evaluated each potential combination of these elements under controlled conditions. This technique identified the best sources of metal, carbon and nitrogen to increase lactoferrin synthesis. The results of the experiment have clarified the relative importance of each component, thus further improving the manufacturing process. Through in-depth analysis of the results, this work aimed to create an evolutionary and economic formulation for the successful synthesis of lactoferrin in *P. pastoris*.

### 2.2.11 5L Bioreactor Fed-Batch Fermentation

#### 2.2.11.1 Equipment and Accessories

| Equipment | Purpose |
|---|---|
| 5-Liter Fermenter (T&J Intelli-Ferm A/B/Q series bioreactor) | For large-scale fermentation |
| pH Electrode | Monitoring pH levels during fermentation |
| DO (Dissolved Oxygen) Electrode | Monitoring oxygen levels during fermentation |
| Sterile Gauze | To prevent contamination during medium preparation |
| Feed Bottles | For methanol and nutrient feeds |

| | |
|---|---|
| Silicone Tubing | For fluid transport |
| Antifoam Agent | To prevent excessive foaming |
| Air Filters | To ensure sterile air supply |
| Syringes and Sampling Tubes | For sampling fermentation broth |
| Clamps and Alcohol Wipes | For securing and cleaning equipment |
| Erlenmeyer Flasks | For collecting and processing gas samples |

2.2.11.2 Operation Process

The seed culture preparation began by thawing a cryopreserved *P. pastoris* strain (Strain No. 5), which was then inoculated into 5 mL YPD liquid medium. The inoculum was incubated at 30°C, with shaking at 200 rpm, for 12-16 hours until the $OD_{600}$ reached ~5, indicating healthy yeast growth. Following this, a loop from the YPD culture was streaked onto YPD solid medium and incubated at 30°C for 3 days to isolate single colonies. A single colony was selected and inoculated into 5 mL GPD liquid medium containing 200 mg/L G418 to promote growth. The culture was again incubated at 30°C, shaking at 200 rpm, for another 12-16 hours until the $OD_{600}$ reached 4-6. After this, the culture was expanded by transferring 3% of the inoculum into 30 mL GPD liquid medium, where it was incubated until it reached an OD600 of 4-6. To ensure sufficient biomass for the fermentation process, the culture was further expanded by transferring 3% of the culture into 50 mL GPD liquid medium in 10 separate flasks. These cultures were grown to serve as the seed inoculum for the fermentation process. Before the fermentation process began, the fermenter and its associated systems underwent a series of preparation steps. First, the equipment, including the 5-liter fermenter, sensors, and nutrient lines, was checked to ensure proper functionality and cleanliness. Calibration of the pH electrode was done before sterilization, and the DO (dissolved oxygen) electrode was calibrated after sterilization to ensure accurate monitoring during the fermentation process. The fermenter and its components, including the sample tubes, air tubing, and other exposed parts, were autoclaved at 121°C for 20 minutes under high pressure to sterilize the system. After the sterilization process, the fermenter was reassembled, ensuring that sterile air was introduced into the system to maintain sterile conditions throughout the fermentation process. Once the fermenter setup was complete, the fermentation process was initiated by inoculating the fermenter with 10% (w/v) of the prepared seed culture (~60 g of wet biomass) into 3L BMMY medium. The pH of the medium was adjusted to 5 ± 0.05 using a 25-30% ammonia solution, and the DO level was maintained above 3.6% by controlling the aeration rate. The fermenter was set to 220 rpm and incubated at 30°C to

facilitate the yeast growth and recombinant protein production. Methanol was added to the fermenter at a concentration of 1% (v/v) every 24 hours to induce protein expression. This induction period continued for 96 hours, and every 24 hours, the medium was replenished with additional methanol to maintain optimal induction conditions. The $FeCl_3$ was added to the fermenter in a single dose to a final concentration of 0.1 g/L to enhance protein production. During the fermentation, samples were collected every 24 hours to measure $OD_{600}$, monitor biomass accumulation, and evaluate protein concentration. Upon completion of the fermentation process, the agitation of the fermenter was stopped, and final data on biomass, protein concentration, and $OD_{600}$ were recorded. The fermentation broth was harvested for downstream processing, such as protein purification. All fermentation data, including $OD_{600}$, protein yield, and other critical parameters, were documented and stored for analysis and future optimization. After harvesting, the fermenter and associated equipment were thoroughly cleaned. The fermenter, electrodes, and other accessories were cleaned using sterile solutions to prevent any potential cross-contamination in subsequent fermentation runs. Routine maintenance and calibration of the sensors were performed to ensure accuracy for future experiments.

# Chapter 3: Results and discussion

## 3.1 Development and Functional Validation of a Recombinant Human Lactoferrin Expression System

### 3.1.1 Construction and Screening of Recombinant Human Lactoferrin-Expressing *P. pastoris*

The hLF gene (NM-002343.2) was synthesized at GenScript Biotech Co., Ltd., amplified with the synthetic gene as a template, obtained the recombinant plasmid by seamless cloning technology and the plasmid PpIC9K with shore resistance through *Eco*R I and *Not* I double cutter, introduced it into DH5α, and then introduced into *P. pastoris* competent state by electroporation, and coated on a plate containing ampicillin (Fig. 3-1). Then pick a single colony and inoculate it into liquid culture medium containing ampicillin resistance. Then extract the recombinant plasmid and use it as a template for amplification. Verify the PCR product by agarose gel electrophoresis and recover it. Fig. 3-2A showed that the recombinant plasmid expressing hLF was constructed in *E. coli* DH5α. Finally, the recombinant plasmid with expressing hLF was introduced into *P. pastoris* competent state by electroporation, Fig. 3-2B showed that the recombinant strain expressing hLF was constructed in *P. pastoris.*

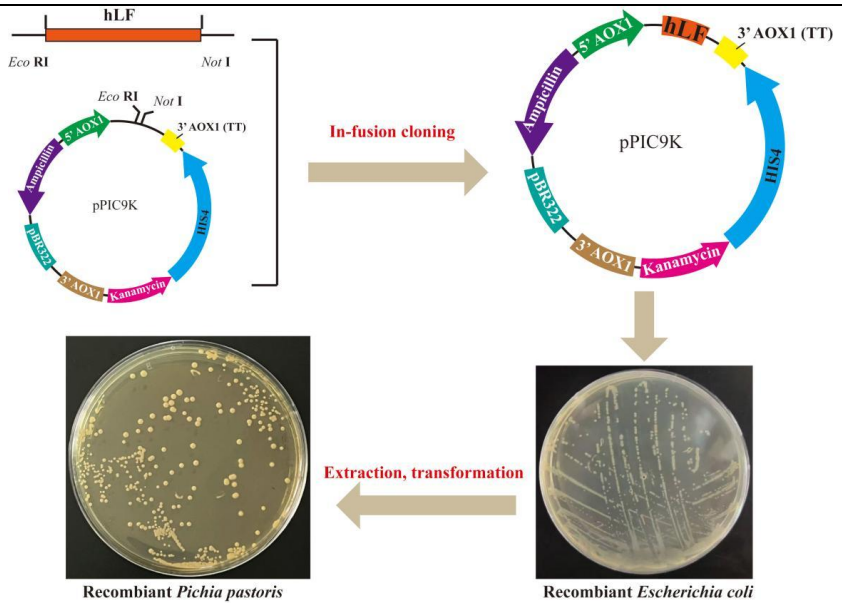



**Fig. 3-1: Construction and verification of recombinant plasmids and strains**

The plasmid diagram illustrates the recombinant *P. pastoris* strain designed to express human lactoferrin (hLF). The hLF gene was included into the expression vector pPIC9K, which includes critical regulatory components such as the AOX1 promoter for methanol-induced expression, a polyadenylation signal for appropriate transcription termination, and a selectable marker for antibiotic resistance.

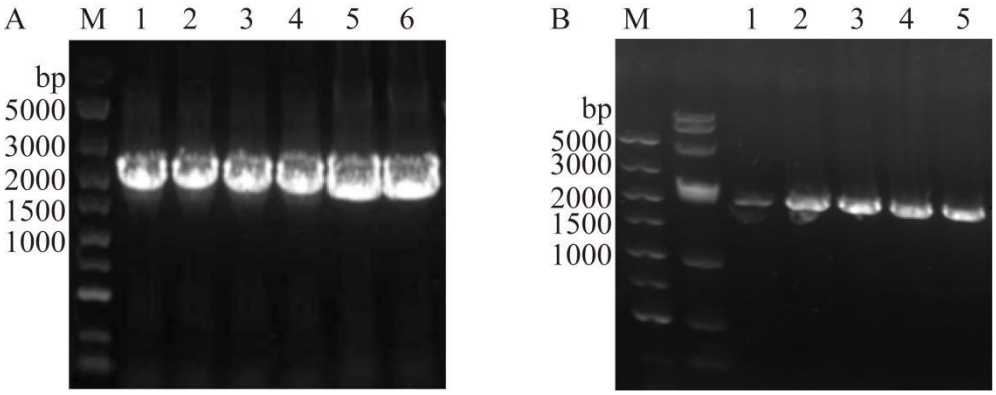



**Fig. 3-2: Construction and verification of recombinant plasmids and strains.**

(A) The electrophoretic figure illustrates the PCR amplification of the hLF gene from the recombinant plasmid pPIC9K-hLF. The enhanced bands verify the efficient incorporation of the hLF gene into the plasmid. DNA markers (M1: 5000 bp and M2: 10,000 bp) used as size benchmarks to confirm the anticipated size of the PCR output. (B) The electrophoresis findings of hLF amplified from individual colonies demonstrate the precision of the transformation process. Several individual colonies (lanes 1-5) were examined by colony PCR to verify the existence of the recombinant plasmid. The control sample (pPIC9K-hLF plasmid) functions as a positive reference. The uniform band patterns in the electrophoresis gel confirm the effective creation of recombinant *P. pastoris*

strains containing the hLF gene.

### 3.1.2 SDS-PAGE and Densitometric Analysis of Recombinant Human Lactoferrin Eexpression in *P.pastoris*

After activating the engineered strain expressing human lactoferrin preserved in the laboratory, it was streaked onto a YPD solid plate, and a single colony was picked and cultured in a YPD liquid test tube. After one day, it was inoculated into a shake flask for culture, and methanol induced protein expression. The qualitative detection method of lactoferrin usually uses biochemical methods. This study used SDS-PAGE to determine the expression of lactoferrin in the supernatant of the fermentation broth, and used spectrophotometry to quantitatively determine the lactoferrin production. The change in bovine serum protein concentration was designed as a guarantee curve, and finally it was clear that methanol induced lactoferrin, and the constructed engineered bacteria were used to express lactoferrin.

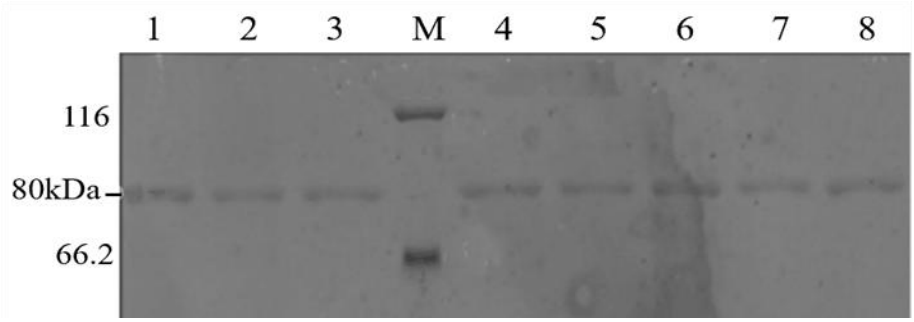



**Fig. 3-3: SDS-PAGE electrophoresis results of fermentation supernatant**

The lanes are designated for the following strains: Lane 1: 2GS115, Lane 2: 3200-2-2, Lane 3: (unspecified), Lane 4: 2-800-1-2, Lane 5: 1-3200-2-2, Lane 6: 1-800-1-2, Lane 7: 1-3200-2, and Lane 8: 1GS115. The lane labeled "M" denotes the protein marker with a typical molecular weight range of 14.4 to 116 kDa. The typical molecular weight of human lactoferrin is around 80 kDa.

### 3.1.3 Antimicrobial activity assay of recombinant human lactoferrin expressed in *P. pastoris*

The antibacterial efficacy of lactoferrin produced in *P. pastoris* was assessed against three representative microorganisms: *S. aureus* (Gram-positive), *E. coli* (Gram-negative), and yeast GS115 (eukaryotic). The inhibitory zone experiment was performed to assess the antimicrobial activity of the lactoferrin-containing fermentation supernatant, utilizing bacterial solutions diluted to equivalent quantities, as shown by the $OD_{600}$ values presented in Table 3-1. Lactoferrin demonstrated a significant inhibitory effect on *S. aureus*, forming visible inhibition zones around the Oxford cups as showed in the Fig. 3-4A. The observed inhibition highlights lactoferrin’s capacity to target and disrupt the bacterial cell

wall or metabolic processes of Gram-positive organisms. This result underscores its potential application in combating Gram-positive bacterial infections.

The inhibition zones for *E. coli* were narrower compared to *S. aureus*, indicating a significantly lesser antibacterial activity in Fig. 3-4B. Depicts the inhibition zones for *E. coli*, a Gram-negative bacterium. The smaller zones suggest comparatively reduced activity due to the protective outer membrane of Gram-negative bacteria. This diminished impact may be attributable to the distinctive outer membrane of Gram-negative bacteria, which functions as an extra protective barrier, reducing the availability and efficiency of antimicrobial compounds like lactoferrin.

The most significant inhibitory impact was noted against yeast GS115, exhibiting bigger inhibition zones than those reported with bacterial strains. Fig. 3-4C The larger zones in this panel demonstrate lactoferrin's potent activity against eukaryotic cells.

This discovery indicates that lactoferrin's antibacterial properties extend to eukaryotic microbes, potentially compromising their cell membranes or affecting vital biological activities.

Table 3-1: Measured $OD_{600}$ after dilution of the test bacterial suspension

| Strains | Dilution multiple | $OD_{600}$ (mean) |
|---|---|---|
| *E. coli* | 6 | 0.567 |
| *S. aureus* | 30 | 0.623 |
| *P. pastoris* | 20 | 0.581 |

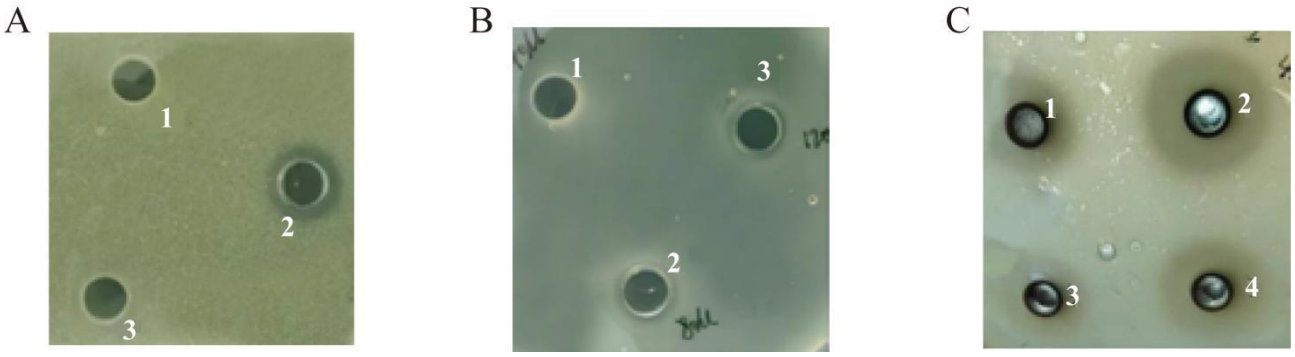



**Fig. 3-4: Antibacterial effect of lactoferrin-containing fermentation supernatant**

(A) Antibacterial Effect on *S. aureus*. (B) Antibacterial Effect on *E. coli*. (C) Antibacterial Effect on Yeast (GS115).

The numbers 1 to 4 in Figure 3-4 represent samples collected on consecutive fermentation days

(Day 1 to Day 4) under the same culture and induction conditions. Each sample was tested for antimicrobial activity to evaluate changes in bioactivity of lactoferrin over time.

The disparity in inhibitory zones among the examined microorganisms indicates variations in lactoferrin's interaction with their cellular architectures and growth patterns. The heightened inhibition seen in yeast may be associated with lactoferrin's superior capacity to damage eukaryotic membranes. The inhibition of *S. aureus* indicates its vulnerability to the antibacterial properties of lactoferrin, including iron chelation and membrane disruption. The reduced inhibition zone for *E. coli* indicates that its Gram-negative membrane provides partial resistance to the activities of lactoferrin. The findings validate that lactoferrin produced in *P. pastoris* maintains its antibacterial efficacy, successfully suppressing the proliferation of several pathogens. This highlights its potential use in antimicrobial therapy, food preservation, and other sectors necessitating natural and effective antimicrobial agents.

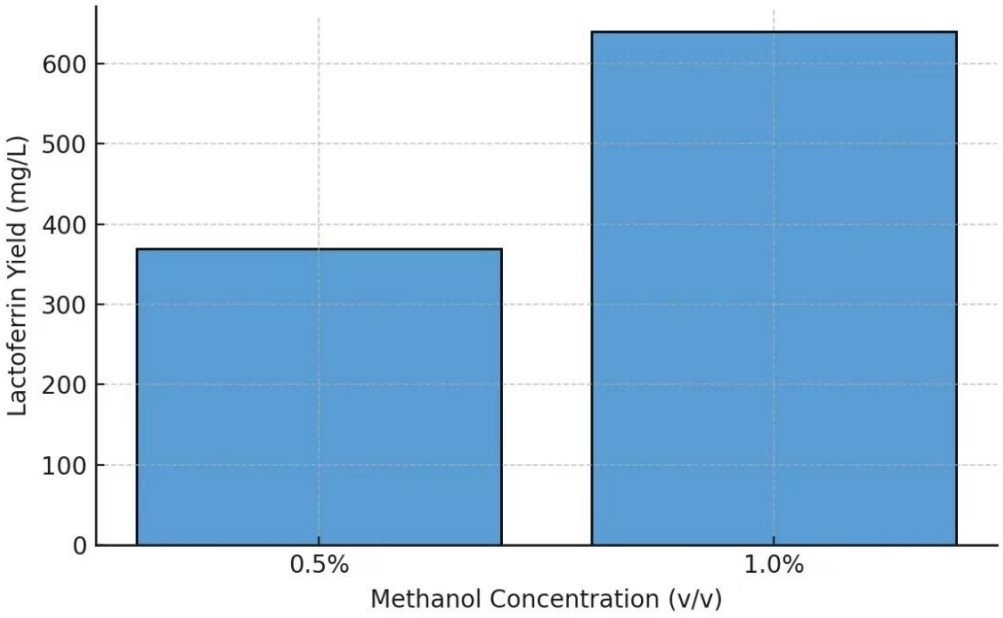
The results establish a foundation for more investigation into enhancing lactoferrin's antibacterial effectiveness against a wider array of infections.

## 3.2 Optimization of Recombinant Human Lactoferrin Expression Conditions in *P. pastoris*

### 3.2.1 Optimization of Methanol Inducer Concentration

To enhance the secretory expression of lactoferrin in recombinant P. pastoris, this study first investigated the effects of different methanol concentrations as inducers on protein expression. Recombinant P. pastoris strains were induced under identical conditions with final methanol concentrations of 0.5% and 1%, respectively. At the endpoint of fermentation, the expression levels of lactoferrin in the culture supernatant were analyzed by SDS-PAGE. As shown in Fig. 3-6, Lanes 1-4 represent lactoferrin expression under 1% methanol induction, while Lanes 5-7 correspond to 0.5% methanol induction. The results clearly demonstrate that the target band intensity (lactoferrin) was higher under 1% methanol induction. Consequently, subsequent experiments were conducted using 1% methanol as the optimized inducer concentration for protein expression.

The induction of recombinant protein expression in *P. pastoris* is predominantly regulated by the AOX1 promoter, which is strongly induced in the presence of methanol. In this study, two methanol concentrations (0.5% and 1.0% v/v) were tested to determine their effects on lactoferrin expression. The results demonstrated a marked increase in protein yield under 1.0% methanol, suggesting that a higher inducer concentration enhances promoter activity and subsequent transcription levels. Methanol serves




not only as a carbon source but also as a metabolic signal that modulates the transcription of methanol-inducible genes. The AOX1 promoter is upregulated at higher methanol levels, which in turn stimulates higher levels of mRNA transcripts encoding lactoferrin. However, concentrations above 1% are often avoided due to their cytotoxic effects, such as the accumulation of formaldehyde and hydrogen peroxide. Hence, 1.0% v/v methanol provided an optimal balance between induction strength and cellular viability.

**Figure. 3-5: Effect of Methanol Concentration on Lactoferrin Expression**

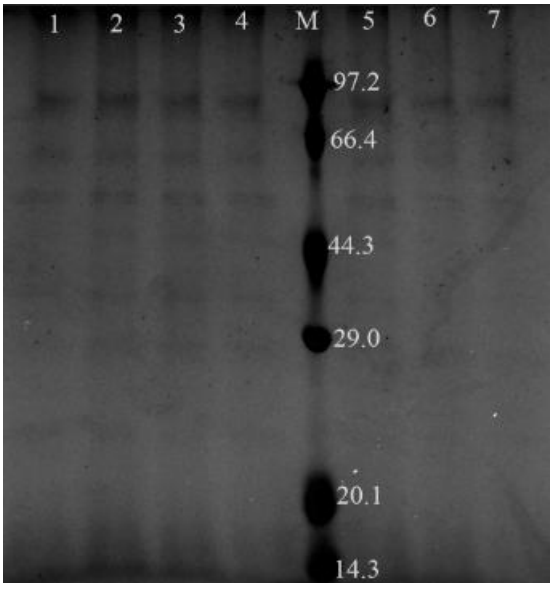



**Fig. 3-6: Effects of different methhenol concentrations as inducers on lactoferrin expression.**

Line 1-4: lactoferrin expression under 1% methanol induction, Lanes 5-7: lactoferrin expression under 0.5% methanol induction.

### 3.2.2 Optimization of $Fe^{3+}$supplementation concentration

Ferric ions ($Fe^{3+}$) influence both the expression and bioactivity of lactoferrin. Therefore, based on the previously determined optimal inducer concentration (Section 3.2.1), this study further investigated the effects of different $Fe^{3+}$ concentrations on lactoferrin production in recombinant *P. pastoris*. Recombinant *P. pastoris* strains were induced under identical conditions with $Fe^{3+}$ supplementation at 0.05 g/L and 0.1 g/L, respectively. At the endpoint of fermentation, lactoferrin expression levels in the culture supernatant were analyzed by SDS-PAGE. As shown in Fig. 3-8, Lanes 1-8 correspond to lactoferrin expression under 0.1 g/L $Fe^{3+}$supplementation, while Lanes 9-14 represent results from 0.05 g/L $Fe^{3+}$ supplementation. The target lactoferrin band intensity was significantly higher under 0.1 g/L $Fe^{3+}$conditions. Consequently, combined with the optimal methanol inducer concentration (1%) determined in Section 3.2.1, the finalized experimental conditions were set as follows: methanol inducer concentration: 1%, $Fe^{3+}$ supplementation: 0.1 g/L. Iron ions ($Fe^{3+}$) are critical cofactors for the proper folding and bioactivity of iron-binding proteins such as lactoferrin. In this experiment, $Fe^{3+}$ supplementation at 0.05 g/L and 0.1 g/L was evaluated to investigate its effect on expression and antimicrobial functionality. SDS-PAGE analysis and inhibition zone assays confirmed that 0.1 g/L $Fe^{3+}$ resulted in a higher protein yield and greater bioactivity. The presence of $Fe^{3+}$ enhances the structural conformation of lactoferrin by facilitating iron binding at its active sites, which is essential for its antimicrobial function. A properly folded lactoferrin molecule can sequester free iron, limiting its availability to pathogens and thereby inhibiting their growth. Furthermore, iron ions influence metabolic pathways related to oxidative phosphorylation and protein biosynthesis in P. pastoris, indirectly supporting higher recombinant protein output. Therefore, $Fe^{3+}$ at 0.1 g/L not only optimized yield but also preserved the functional integrity of the expressed protein.

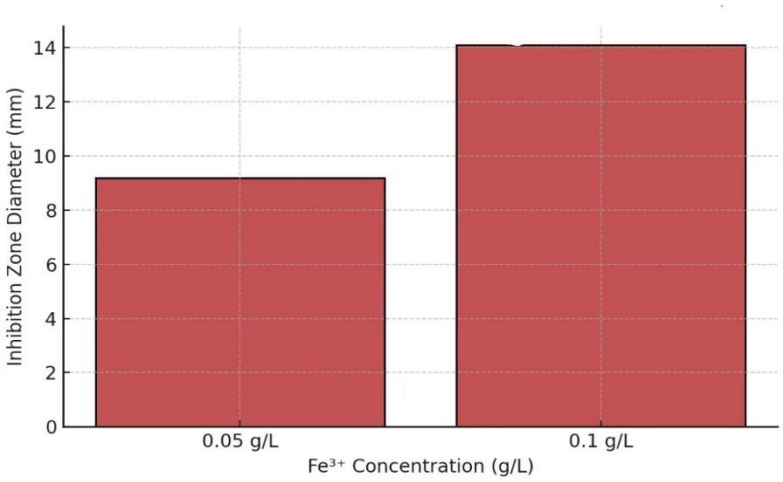



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**Fig. 3-7: Effect of $Fe^{3+}$ Concentration on Antimicrobial Activity of Lactoferrin**

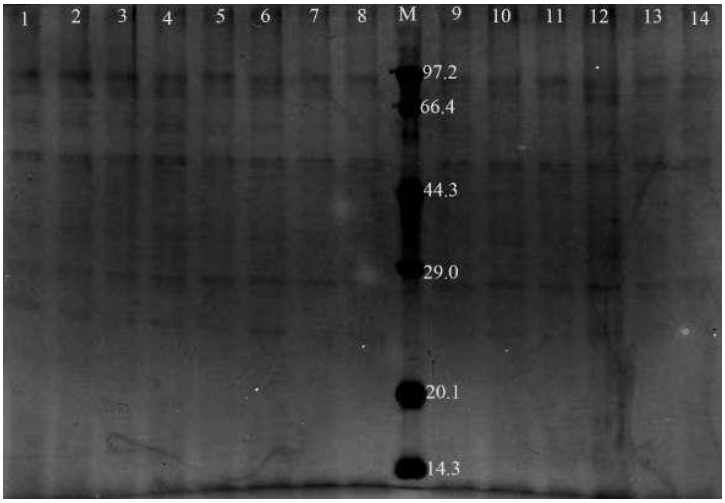


**Fig. 3-8: Effects of Different $Fe^{3+}$ concentrations on lactoferrin expression**

Line 1-8: Lactoferrin expression under 0.1 g/L $Fe^{3+}$ supplementation, Lanes 9-14: Lactoferrin expression under 0.05 g/L $Fe^{3+}$ supplementation.

### 3.2.3 Bovine serum albumin (BSA) standard curve determination

The concentration of the lactoferrin-containing fermentation supernatant was measured using the BSA (Bradford) method. A series of bovine serum albumin (BSA) standard solutions with varying concentrations were prepared according to the dilution protocol outlined in the table. Using a 1 mg/mL BSA stock solution as the standard, a standard curve was generated by plotting the

absorbance values against the corresponding BSA concentrations. The prepared standard curve is shown in the Fig. 3-9. The absorbance values of the unknown lactoferrin samples were then substituted into the standard curve equation to calculate their concentrations.

Table 3-2: Bovine serum albumin (BSA) dilution concentration

| Regents | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| $ddH_2$ (μL) | 100 | 97.5 | 95 | 92.5 | 90 | 87.5 | 85 | 80 |
| 1mg/ml BSA (μL) | 0 | 2.5 | 5 | 7.5 | 10 | 12.5 | 15 | 20 |
| BSA (μg/ml) | 0 | 2.5 | 5 | 7.5 | 10 | 12.5 | 15 | 20 |
| Real concentration | 0.665 | 0.703 | 0.743 | 0.775 | 0.811 | 0.851 | 0.878 | 0.936 |

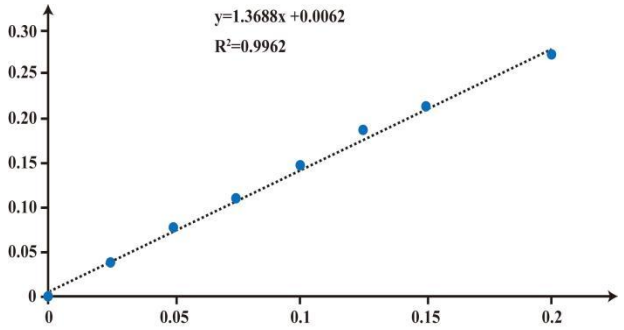


**Fig. 3-9: Bovine serum albumin (BSA) standard curve determination.**

### 3.2.4 Orthogonal Experiment Optimization of Fermentation Medium

The experimental design was designed using the L16 ($4^3$) orthogonal test table as showed in table 3-3, allowing for an efficient examination of the three components at four levels each. The parameters evaluated included different carbon sources, nitrogen sources, and metal ions, each tested to assess their influence on lactoferrin synthesis in *P. pastoris*. Protein content in the resultant fermentation supernatants was evaluated using the Coomassie Brilliant Blue assay, with the standard curve equation constructed as y=1.3688x+0.0062, $R^2$=0.9962 (Fig. 3-9). This achieved great precision in protein measurement and allowed for exact comparisons between experimental conditions.

Table3-3: Orthogonal factor experimental level table

| level | Factor | | |
|---|---|---|---|
| | Carbon source(g/L) | Nitrogen source(g/L) | Metal(g/L) |
| 1 | glycerin(1) | Yeast powder-$(NH_4)_2SO_4$(10+20) | $MnCl_2$(0.1) |
| 1 | glycerin(1) | Yeast Powder-Peptone(10+20) | $CuCl_2$(0.1) |
| 1 | glycerin(1) | Yeast extract powder-fish peptone(10+20) | $FeCl_3$(0.1) |
| 1 | glycerin(1) | Soybean cake powder-beef extract(10+20) | $ZnCl_2$(0.1) |
| 2 | glucose(1) | Yeast powder-$(NH_4)_2SO_4$(10+20) | $ZnCl_2$(0.1) |
| 2 | glucose(1) | Yeast Powder-Peptone(10+20) | $FeCl_3$(0.1) |
| 2 | glucose(1) | Soybean cake powder-beef extract(10+20) | $MnCl_2$(0.1) |
| 2 | glucose(1) | Yeast extract powder-fish peptone(10+20) | $CuCl_2$(0.1) |
| 3 | Corn Syrup(1) | Yeast extract powder-fish peptone(10+20) | $MnCl_2$(0.1) |
| 3 | Corn Syrup(1) | Yeast Powder-Peptone(10+20) | $ZnCl_2$(0.1) |
| 3 | Corn Syrup(1) | Soybean cake powder-beef extract(10+20) | $CuCl_2$(0.1) |
| 3 | Corn Syrup(1) | Yeast powder-$(NH_4)_2SO_4$(10+20) | $FeCl_3$(0.1) |
| 4 | water(1) | Soybean cake powder-beef extract(10+20) | $FeCl_3$(0.1) |
| 4 | water(1) | Yeast Powder-Peptone(10+20) | $MnCl_2$(0.1) |
| 4 | water(1) | Yeast powder-$(NH_4)_2SO_4$(10+20) | $CuCl_2$(0.1) |
| 4 | water(1) | Yeast extract powder-fish peptone(10+20) | $ZnCl_2$(0.1) |

This experiment assessed the impact of various carbon, nitrogen, and metal sources on recombinant lactoferrin synthesis in *P. pastoris*. The data displayed in the table above demonstrate the protein yields (mg/L) achieved across 16 experimental configurations utilizing diverse combinations of these parameters. Every serial number is associated with a distinct mix of carbon source, nitrogen source, and metal. The K values (K1, K2, K3, K4) and their respective k averages elucidate the impact of each variable. The absolute value of R represents the degree of influence of different factors on lactoferrin production, and the larger the R value, the greater the influence of this factor. The nitrogen supply (R=147.5) was the predominant factor in protein formation, succeeded by the carbon source (R=100), whilst the metal source (R=20) exerted minimal influence. Look directly at the results , the setup 4 attained the maximum protein content, utilizing glycerin (carbon source 1), soybean cake powder-beef extract (nitrogen source 3), and Fecl3 (metal source 3), resulting in 640 mg/L, which was 2 times higher than the protein content of the original culture medium. These findings underscore the necessity to optimize nitrogen and carbon supplies to get optimal protein output, whereas metal sources seem to exert negligible impact under the examined conditions.

Table 3-4: Orthogonal design and results

| serial number | Carbon source | Nitrogen source | Metal | Protein content (mg/L) |
|---|---|---|---|---|
| 1 | 1 | 4 | 4 | 130 |
| 2 | 1 | 1 | 1 | 300 |
| 3 | 1 | 2 | 2 | 260 |
| 4 | 1 | 3 | 3 | 640 |
| 5 | 2 | 4 | 3 | 70 |
| 6 | 2 | 1 | 2 | 190 |
| 7 | 2 | 3 | 4 | 610 |
| 8 | 2 | 2 | 1 | 250 |
| 9 | 3 | 2 | 4 | 230 |
| 10 | 3 | 1 | 3 | 150 |
| 11 | 3 | 3 | 1 | 570 |
| 12 | 3 | 4 | 2 | 130 |
| 13 | 4 | 3 | 2 | 540 |
| 14 | 4 | 1 | 4 | 150 |

| | | | | |
|---|---|---|---|---|
| 15 | 4 | 4 | 1 | 30 |
| 16 | 4 | 2 | 3 | 210 |
| K1 | 1330 | 790 | 1150 | |
| K2 | 1120 | 950 | 1120 | |
| K3 | 1080 | 2360 | 1070 | |
| K4 | 930 | 360 | 1120 | |
| k1 | 332.5 | 197.5 | 287.5 | |
| k2 | 280 | 237.5 | 280 | |
| k3 | 270 | 590 | 267.5 | |
| k4 | 232.5 | 90 | 280 | |
| R | 100 | 147.5 | 20 | |

The results highlight the essential importance of nitrogen supplies in augmenting lactoferrin synthesis and offer a definitive pathway for future culture medium modification. By concentrating on combinations that enhance nitrogen source efficacy, researchers may substantially increase protein yields in recombinant *P. pastoris* systems.

The analysis of variance (ANOVA) for the regression model elucidates the principal parameters affecting recombinant lactoferrin production in *P. pastoris*. The findings, outlined in Table 3-5, illustrate the importance of the examined factors, such as carbon sources (C Source), nitrogen sources (N Source), and metals, on protein production. The regression model is very significant, with a p-value below 0.0001, demonstrating that the chosen parameters significantly influence lactoferrin synthesis. The nitrogen source demonstrates the most significant impact among the various components, evidenced by its high F-value of 135.72 and an exceptionally low p-value of less than 0.0001. This discovery highlights the essential need of choosing the suitable nitrogen source to optimize lactoferrin production. The carbon source is statistically significant, exhibiting an F-value of 4.92 and a p-value of 0.0467, underscoring its relevance in enhancing production efficiency. In contrast, the metal source exerts an insignificant influence on protein synthesis, demonstrated by its low F-value of 0.1988 and a p-value of 0.8936, indicating that changes in metal source composition are improbable to substantially effect lactoferrin yields in experiment conditions. The residual sum of squares (8300.00) and mean square (1383.33) suggest a strong model fit, since the regression adequately accounts for the observed variability in protein synthesis. The results offer critical insights for prioritizing nitrogen and carbon sources as essential optimization objectives in the formulation of cost-effective and efficient culture medium for lactoferrin synthesis.

Table 3- 5: Analysis of variance for regression models

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| Model | 5.845 | 9 | 64941.67 | 46.95 | <0.0001 | Significant |
| C Source | 20425.00 | 3 | 6808.33 | 4.92 | 0.0467 | |
| N Source | 5.632 | 3 | 1.87741 | 135.72 | <0.0001 | |
| Metal | 825.00 | 3 | 275.00 | 0.1988 | 0.8936 | |
| Residual | 8300.00 | 6 | 1383.33 | | | |
| Cor Total | 592775 | 15 | | | | |

### 3.2.5 Scale-up Production of Lactoferrin in a 5-Liter Fermenter

This study utilized the recombinant *P. pastoris* strain constructed and validated in Section 3.1 as the starting strain. Recombinant human lactoferrin was expressed through stepwise seed culture amplification and methanol induction. The recombinant *P. pastoris* was first inoculated into 5 mL of YPD liquid medium (30℃, 200 rpm, 12-16 hours). Single colonies were isolated by streaking and then transferred to GPD medium containing 200 mg/L G418 for expansion until $OD_{600}$ reached 4-6. Subsequently, the culture was scaled up stepwise at a 3% inoculation ratio into 30 mL and 50 mL GPD medium to obtain a high-density seed culture (total inoculum ≈60 g wet biomass). Under sterile conditions, the seed culture was inoculated into the fermentation medium at a 10% inoculation ratio to initiate fermentation. During the fermentation, 1% (v/v) methanol was supplemented every 24 hours for induction (4 cycles, total 96 hours), with $FeCl_3$ (final concentration 0.1 g/L) added simultaneously to enhance protein expression. Samples were collected every 24 hours to measure $OD_{600}$, monitor biomass accumulation, and evaluate protein concentration. As shown in Figure 3-8, the cell density of the recombinant strain reached the stationary phase by 36 hours as fermentation progressed, while lactoferrin production gradually increased to its peak. The highest lactoferrin yield reached 693.94mg/L at 48 hours of fermentation.

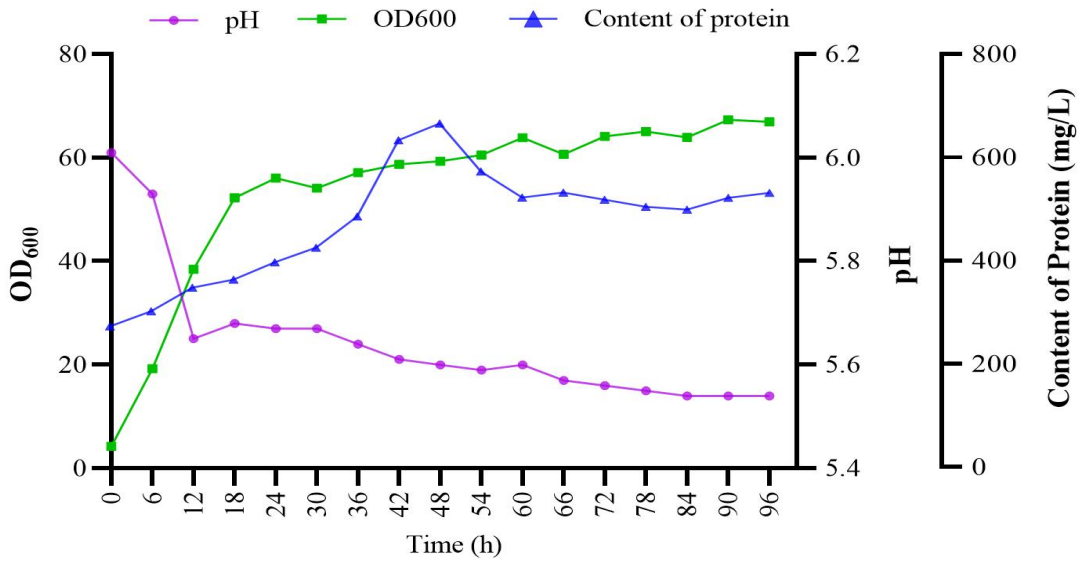


**Fig. 3-10 Protein content variation with stain concentration.**

## Chapter 4: Conclusion and Prospects

### 4.1 Conclusion

Compared to traditional extraction techniques, this work demonstrated that *P. pastoris* offers a powerful expression platform for the synthesis of recombinant human lactoferrin (rhLF), with notable advantages. Using the pPIC9K expression system to integrate the hLF codon-optimized gene into the GS115 genome, we were able to create a viable recombinant strain through meticulous genetic engineering. The efficient integration and stability of the expression cassette were validated by extensive molecular characterization, including colony PCR, restriction digestion analysis and DNA sequencing. If functional tests have shown its preserved bioactivity, including strong antimicrobial effects against clinically relevant pathogens (*Staphylococcus aureus* and *Escherichia coli*) and the GS115 yeast strain, the SDS-PAGE analysis confirmed the production of full length rhLF, about 80 kDa.

The fermentation process has been rigorously and methodically optimized. We demonstrated, through carefully planned tests, that optimal protein synthesis was achieved while preserving cell viability when 0.1 g/L of $Fe^{3+}$ supplementation was associated with 1% (v/v) methanol induction. Our creative use of an orthogonal experiment plan (L16 matrix) revealed that the best source of carbon was corn syrup (1 g/L) and the best combination of nitrogen sources was soybean meal powder and beef

extract (10-20 g/L). This resulted in a significant increase of 2 times the production of rhLF (570 mg/L) over baseline conditions. The industrial scalability of this expression system has been confirmed by the successful scaling of 5 L bioreactor fermentation, which has produced consistent yields of 693.94 mg/L.

**4.2 Prospects**

Despite the certain progress made in this study, further improvements are necessary to enhance productivity, reduce production costs, and expand the applications of recombinant lactoferrin. One of the primary areas for future research is genetic engineering of *P. pastoris* to further increase lactoferrin expression levels. Enhancing promoter strength, optimizing codon usage, and engineering strains for higher secretion efficiency could significantly improve yield. Additionally, the development of glycoengineered strains capable of producing human-like glycosylation patterns would be beneficial for applications in therapeutic and pharmaceutical fields where precise glycosylation is critical for protein bioactivity. The introduction of co-expression systems with chaperone proteins or other factors that assist in proper folding and stability may also enhance lactoferrin production.

Further optimization of fermentation parameters is another crucial area for future exploration. Investigating alternative induction strategies, such as stepwise methanol feeding, mixed-feed strategies, or alternative inducers, could lead to improved productivity while minimizing methanol toxicity. Additionally, integrating real-time monitoring systems and automated process control could enable better regulation of pH, temperature, dissolved oxygen, and nutrient supply, leading to more consistent fermentation outcomes. Exploring alternative carbon and nitrogen sources that are more cost-effective and sustainable would further reduce production costs while maintaining high yields.

The transition from laboratory-scale to industrial-scale production presents another key challenge. While the 5-liter bioreactor experiments demonstrated the feasibility of scaling up, further studies on larger bioreactors (e.g., 10L, 50L, or industrial-scale fermenters) will be necessary to fully evaluate the potential for commercial production. The use of bioprocess modeling and simulation could aid in predicting large-scale fermentation performance, allowing for more precise optimization of growth conditions. Additionally, refining downstream processing techniques, including filtration, chromatography, and drying methods, will be essential to improve the purity and stability of recombinant lactoferrin while ensuring cost-effectiveness.

Another critical area of focus is the development of applications for recombinant lactoferrin. Research into encapsulation or formulation techniques can improve protein stability and bioavailability, making lactoferrin more suitable for pharmaceutical, food, and nutraceutical applications. Potential

applications include antimicrobial treatments, immune system support, and functional food enrichment, with additional studies needed to evaluate the effects of recombinant lactoferrin in clinical and industrial settings. Investigating the interaction of recombinant lactoferrin with different bacterial strains, viruses, and host cells could further expand its potential use in treating infections, supporting immune function, and even exploring its role in cancer therapy.

Regulatory considerations will also play a significant role in the commercialization of recombinant lactoferrin. Comprehensive safety assessments, toxicological studies, and clinical trials will be necessary to meet the regulatory standards required for pharmaceutical and food applications. Ensuring compliance with agencies such as the FDA, EMA, and CFDA will be crucial in facilitating market entry. Additionally, intellectual property protection and patenting strategies will be important for securing rights to recombinant lactoferrin production methods and ensuring the competitiveness of the technology in the global market.

Finally, the increasing demand for sustainable and scalable biomanufacturing solutions highlights the importance of continued innovation in recombinant protein production. By integrating synthetic biology, metabolic engineering, and machine learning-based optimization, future research could further improve process efficiency, reduce costs, and enhance yield consistency. Exploring alternative host systems, such as plant-based or insect cell expression platforms, could provide additional options for large-scale lactoferrin production with distinct advantages in yield, scalability, and regulatory approval.

In conclusion, this study provides a solid foundation for the large-scale production of recombinant lactoferrin using *P. pastoris*, demonstrating its feasibility and potential for industrial applications. However, continued research and technological advancements will be necessary to further optimize production, reduce costs, and expand the applications f lactoferrin in food, medicine, and biotechnology. By addressing these key areas, the future of recombinant lactoferrin production holds great promise for biopharmaceuticals, functional foods, and global healthcare applications, making this protein more accessible and beneficial for diverse industries

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## Publishing papers and awards during master's degree

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Publications

1. Liu, K., Tong, Z., Zhang, X., Dahmani, M., Zhao, M., Hu, M., Li, X., & Xue, Z. (2024). A Review: Development of a Synthetic Lactoferrin Biological System. BioDesign Research, 6(Article 0040). https://doi.org/10.34133/bdr.0040

Awards and Honors

1. Second Prize in the Anhui University Engineering Innovation Contest (2024)
   Awarded to the undergraduate creative group for the project titled "Business Plan of Protein Synthesis (Wuhu) Biotechnology Co., Ltd."Awarded by: Anhui University,2024 this award recognized the innovative approach to protein synthesis and biotechnology.

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