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Translational Modification and pH Optimization of Expression Media for High-Yield Recombinant Human-like Collagen (RHC) Production in *Pichia pastoris* GS115

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Abstract— *Collagen plays a vital role in maintaining tissue structure and promoting repair, making it a key component of the extracellular matrix. Due to the limitations associated with extracting collagen from animal sources, interest in recombinant human-like collagen (RHC) has grown substantially. This study presents an approach to enhance RHC production in Pichia pastoris GS115 by engineering a novel plasmid (pPIC9K-OSTI/RHC) and refining fermentation parameters. Among the tested conditions, a pH of 6 was found to be optimal, resulting in an RHC high yield of 0.35 g/L. The use of the OSTI α-signal peptide notably improved secretion efficiency, contributing to increased protein output. This work demonstrates a scalable and cost-effective strategy for producing recombinant human-like collagen, laying the groundwork for future applications in tissue engineering, wound healing, and biomedical research.*

Keywords— ***Recombinant Human-Like Collagen (RHC), Pichia pastoris, OSTI signal peptide, pH optimization, Protein expression.***

I. INTRODUCTION

Collagen is a fundamental fibrous protein that serves as a primary structural element within the extracellular matrix of connective tissues such as skin, bone, tendons, and cartilage. As the most abundant protein in the human body, it plays an essential role in preserving tissue architecture, facilitating wound repair, and influencing cellular behaviors, including adhesion, migration, and proliferation [1, 2]. Its stability and biological function are largely attributed to its distinctive triple-helical conformation, which is formed by a repeating amino acid motif $(Gly\text{-}X\text{-}Y)_n$—where glycine is consistently present, and the X and Y positions are commonly occupied by proline and hydroxyproline[3, 4]. This structural arrangement enables collagen to withstand mechanical forces and provide tensile strength to connective tissues[3, 5].

Collagen's exceptional mechanical strength and inherent biocompatibility make it a highly sought-after material for a wide range of biomedical applications, including tissue engineering, wound repair, and drug delivery systems. As the predominant structural protein within the extracellular matrix, collagen is instrumental in facilitating tissue regeneration and healing, particularly within the scope of regenerative medicine[6]. Biomaterials derived from collagen have demonstrated considerable potential not only in the restoration of skin, bone, and cartilage but also in accelerating healing processes by promoting cellular adhesion and proliferation[7].

Conventional methods of extracting collagen from animal-derived tissues present several limitations, including elevated production costs, intricate processing steps, and ethical concerns related to animal welfare and cultural or religious restrictions[8, 9]. The reliance on animal sources introduces moral challenges, especially in communities where the use of specific animal products is prohibited by religious or cultural beliefs [10, 11]. Although collagen can also be synthesized through the cultivation of animal cells, this approach is often hindered by low efficiency,

stemming from the high nutritional requirements of the cultures and the relatively modest yields achieved[8, 12].

Microbial platforms, especially *P. pastoris*, have gained attention as effective systems for producing recombinant collagen, owing to their ability to generate substantial amounts of heterologous proteins. These organisms are non-pathogenic and thrive under relatively simple cultivation conditions, making them attractive for industrial applications[13-15]. *P. pastoris* uniquely combines features of both prokaryotic and eukaryotic cells, supporting rapid proliferation and high-cell-density fermentation—key advantages for scalable protein manufacturing[15].

Fine-tuning the conditions for protein expression is essential to achieving high yields in *P. pastoris*. Utilizing specialized media such as Buffered Minimal Methanol Yeast (BMMY) has been shown to significantly boost protein production, as it supplies essential nutrients while supporting effective methanol induction—critical since methanol serves as a key carbon source for this yeast[16, 17]. Moreover, optimizing fermentation variables like temperature, pH, and nutrient availability can further enhance the synthesis of recombinant proteins in *P. pastoris* systems[17, 18].

This research aims to enhance the production of recombinant human-like collagen (RHC) in *P. pastoris* by optimizing the BMMY expression medium and employing a newly designed plasmid construct (pPIC9K-OSTI/RHC). The primary goal is to determine the most effective fermentation conditions, particularly the optimal pH, for maximizing protein yield. Through systematic refinement of these parameters, the study seeks to improve both the efficiency and scalability of RHC production, presenting a more sustainable and cost-efficient alternative to conventional collagen extraction methods.

II. MATERIALS AND METHODS

2.1 Reagents, Strains, Plasmids, and Culture Media

Primers, STAR GXL DNA polymerase, and restriction enzymes were obtained from Takara Bio (Dalian, China). Geneticin (G418 sulfate), used as a selective antibiotic, was purchased from Sangon Biotech (Shanghai, China), while other reagents were sourced from Sinopharm Chemical Reagent (Shanghai, China). The *P. pastoris* GS115 strain was maintained in our laboratory. The expression plasmid pPIC9K was custom-synthesized and acquired from Sangon Biotech. The yeast extract peptone dextrose (YPD) medium was composed of 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract. Buffered glycerol complex medium (BMGY) included 20 g/L tryptone, 10 g/L yeast extract, 3 g/L K_2HPO_4, 11.8 g/L KH_2PO_4, 13.4 g/L yeast nitrogen base (YNB), and 0.5% (v/v) glycerol. Buffered methanol complex medium (BMMY) shared the same formulation as BMGY, with 0.5% (v/v) methanol added for induction. Minimal dextrose (MD) medium, used for screening histidine auxotrophs, contained 20 g/L glucose, 13.4 g/L YNB, and 20 g/L agar. Solid YPD medium was prepared using 20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, and 20 g/L agar. All media formulations followed the guidelines provided in the Invitrogen PichiaPink™ Expression System manual.

2.2 Expression of RHC in *P. pastoris*

2.2.1 Construction of Recombinant Plasmid

In this study, three recombinant plasmids were developed: the wild-type pPIC9K, pPIC9K-RHC, and pPIC9K-OSTI/RHC. The pPIC9K plasmid served as the unmodified control, containing no inserted protein-coding sequences. The pPIC9K-RHC variant was engineered to include a gene encoding recombinant human-like collagen (RHC). For the third construct, pPIC9K-OSTI/RHC, both the RHC gene and the OSTI gene (accession number NC_001142.9) from Saccharomyces cerevisiae S288C were introduced into the signal peptide's pre-region. The pPIC9K-OSTI/RHC plasmid was synthesized by Sangon Biotech. Comprehensive details regarding the plasmid constructs and their sources are summarized in Table 1. All three plasmids were subsequently expressed in *P. pastoris* GS115 cells.

Table 1: Strain, plasmids, and primers used in this study:

Names	Genotype	References
P. pastoris GS115	His4, host strain	Invitrogen
E. coli Top 10	plasmid-cloning host	Invitrogen
AOX1-F	Forward primer (5'-3'), Seq: GACTGGTTCCAATTGACAAGC	Talen-bio Technology Co., Ltd
AOX1-R	Reverse primer (5'-3'), Seq: GGCAAATGGCATTCTGACA	Talen-bio Technology Co., Ltd

pPIC9K	Plasmid	Sangon Biotech Co., Ltd
pPIC9K-OSTI/RHC	pPIC9K plasmid containing the OSTI gene (NC_001142.9) from Saccharomyces cerevisiae S288C pre-region in signal peptide and optimized RHC.	Sangon Biotech Co., Ltd (This Study)
pPIC9K-RHC	pPIC9K plasmid containing RHC	This study

2.2.2 Yeast Transformation and Screening

The expression plasmid was linearized using the SalI restriction enzyme, and the resulting fragments were purified before being introduced into *P. pastoris* GS115 competent cells via electroporation (1500 V, 200 Ω, 50 μF). Transformed cells were spread onto minimal dextrose (MD) agar plates and incubated at 30 °C for 2–3 days. Colonies were screened using colony PCR with AOX1t-F and AOX1t-R primers to identify successful transformants. To isolate high-copy variants, confirmed pPIC9K-OSTI/RHC-positive colonies were transferred to YPD agar plates containing 3 mg/mL geneticin (G418) and incubated at 30 °C for 48 hours.

2.3 Fermentation Media

2.3.1 Characterization of Fermentation Broth

pH Measurement:

The pH of *P. pastoris* GS115 fermentation was monitored, as it is critical for exogenous protein secretion. Six different pH values (4, 5, 5.5, 6, 6.5, and 7.0) were tested for each culture medium, and the pH was measured every 24 hours until the end of fermentation using a pH meter (Starter 5000 pH ST3100, Changzhou, China)[19].

Optical Density (OD) Measurement:

During the fermentation process, the growth of *P. pastoris* was monitored by measuring the optical density at 600 nm (OD_{600}). For each measurement, 100 μL of the culture broth was diluted with 900 μL of sterile distilled water to obtain a 10-fold dilution. The diluted samples were thoroughly mixed, and the absorbance was measured at 600 nm using distilled water as the blank control, using an ultraviolet spectrophotometer (UVmini-1280, Columbia, USA)[20].

2.3.2 Secretion and Expression of RHC in Shaking Flasks

The recombinant *P. pastoris* GS115 strains carrying the RHC gene were initially cultured in 5 mL YPD medium for 12 hours at 30°C and 220 rpm. A 0.5 mL aliquot of this suspension was then transferred to shaking flasks containing 50 mL BMGY medium, and the culture was grown until the OD_{600} reached approximately 15. The cells were harvested and resuspended in 200 mL of BMMY media to induce RHC expression at 30°C and 220 rpm. Methanol was added to a final concentration of 0.5% (v/v) every 12 hours to induce protein expression[21]. The fermentation supernatant was collected for further identification.

2.4 SDS-PAGE Analysis of RHC

To evaluate the expression of RHC, the fermentation supernatant was harvested by centrifugation (7000 × g, 10 minutes). An 80 μL aliquot of the supernatant was mixed with 20 μL of 5× SDS loading buffer, then heated at 95°C for 10 minutes. The sample was subjected to SDS-PAGE analysis, using a 5% stacking gel and a 15% resolving gel. The gel was stained with Coomassie Brilliant Blue.

2.5 Protein Purification

Protein extraction was carried out using ammonium sulfate precipitation. Fermented cell suspensions from shaker flasks were subjected to centrifugation at 4000 rpm for 30 minutes at 4 °C using a refrigerated high-speed centrifuge to separate the fermentation supernatant. To eliminate non-target proteins, a 20% ammonium sulfate solution was added, followed by centrifugation to remove the resulting precipitate. Subsequently, a 60% ammonium sulfate concentration was used to precipitate the RHC protein selectively. The collected protein pellet was then dissolved in ultrapure water. Protein concentration in the resulting supernatant was quantified using the bicinchoninic acid (BCA) assay.

III. RESULTS AND DISCUSSION

3.1 Plasmid Construction and Gene Expression

In this study, we constructed three distinct plasmids: pPIC9K, pPIC9K-RHC, and pPIC9K-OSTI/RHC, as shown in Figure 1. To confirm the proper expression of these genes, we performed a PCR experiment. Figure 2(a) displays the band intensity for both the pPIC9K-RHC and pPIC9K-OSTI/RHC genes, which correspond to 10,041 bp and 10,050 bp, respectively. Figure 2(b) shows the plasmid region containing the RHC protein, which spans 1,157 bp. We used AOX1-F and AOX1-R primers to amplify and identify the desired bands during the PCR screening. The band intensity results confirm that the pPIC9K-RHC and pPIC9K-OSTI/RHC plasmids were successfully constructed. Since the pPIC9K plasmid is a wild-type

version without the RHC protein, we omitted the PCR test for this plasmid.

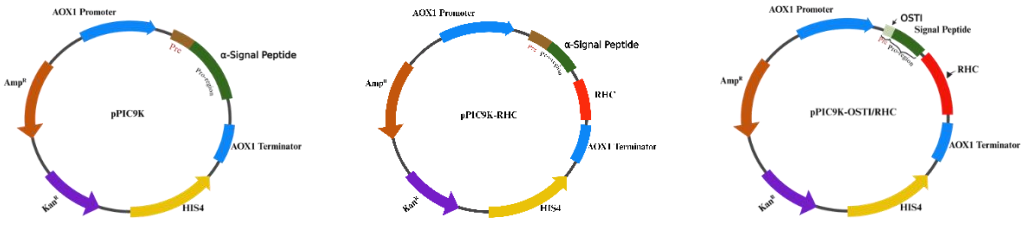

Fig.1: Construction of three different pPIC9K plasmids.

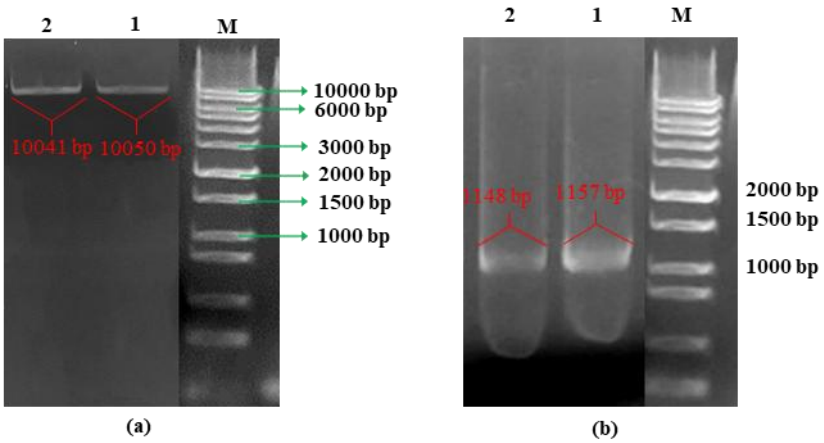

Figure 2: PCR screening: Showing the total base pairs of the whole plasmid. Lane M: 1kb Plus DNA Ladder marker, Lane 1: pPIC9K-OSTI/RHC, Lane 2: pPIC9K-RHC. (a); Showing the base pairs between the AOX1 promoter and AOX1 terminator. Lane M: 1kb Plus DNA Ladder marker, Lane 1: pPIC9K-OSTI/RHC, Lane 2: pPIC9K-RHC. (b)

3.2 Effect of OSTI α-Signal Peptide

Figure 3(a) illustrates the growth of three plasmid variants over time, with the OD_{600} values plotted against time. The growth of pPIC9K-RHC is slightly higher than that of the other variants. Although the first two plasmids show higher growth initially, our desired plasmid, pPIC9K-OSTI/Hss, exhibits superior growth over time. Figure 3(b) confirms that pPIC9K-OSTI/RHC shows the highest band intensity. The protein was identified with a molecular weight of 27 kDa, which conforms with the findings of Ma et al.[22].

The observed improvement in protein secretion is primarily due to substituting the α-factor pre-region with the OSTI signal peptide derived from Saccharomyces cerevisiae S288C, which enhances the secretion efficiency of recombinant RHC. This signal peptide facilitates co-translational translocation into the endoplasmic reticulum (ER), promoting more efficient protein processing and expression. Such a mechanism is particularly beneficial for the secretion of structurally complex proteins[23], as it minimizes the likelihood of misfolding and intracellular aggregation, as demonstrated in previous studies[24]. Incorporation of the OSTI signal sequence into the recombinant plasmid pPIC9K-OSTI/RHC significantly boosts protein levels in the culture supernatant, streamlining downstream purification and improving overall production efficiency[25].

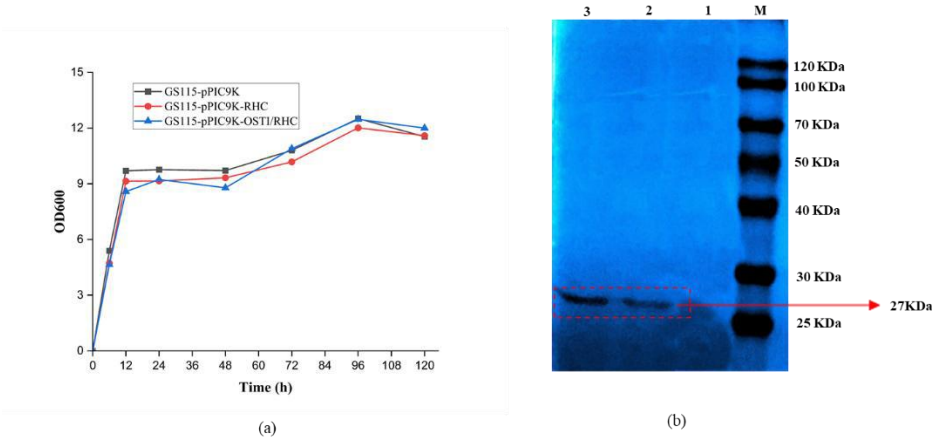

Figure 3: Analysis of the growth of three different P. pastoris GS115. (a) SDS-PAGE analysis of three different plasmids. Lane M (Protein Marker Blue Plus II), Lane 1 (pPIC9K), Lane 2 (pPIC9K-RHC), and Lane 3 (pPIC9K-OSTI/RHC). (b)

3.3 Construction of High-Copy Transformants

Figure 5 illustrates the construction of high-copy transformants using the pPIC9K-OSTI/RHC plasmid. To assess the plasmid's growth under different conditions, we treated it with and without an antibiotic and monitored its growth over time. The OD600 value for the pPIC9K-OSTI/RHC with antibiotic reached 13.14 after 96 hours, while the OD600 for the plasmid without antibiotic was 12.01 (Figure 6a). This indicates that the presence of the antibiotic promotes higher growth of the pPIC9K-OSTI/RHC plasmid. To further confirm protein expression in *P. pastoris* G115, we performed an SDS-PAGE analysis for both conditions (Figure 6b). The results from the SDS-PAGE analysis are consistent with our observations, showing a higher band intensity for the pPIC9K-OSTI/RHC with antibiotic treatment compared to the same construct without antibiotic treatment.

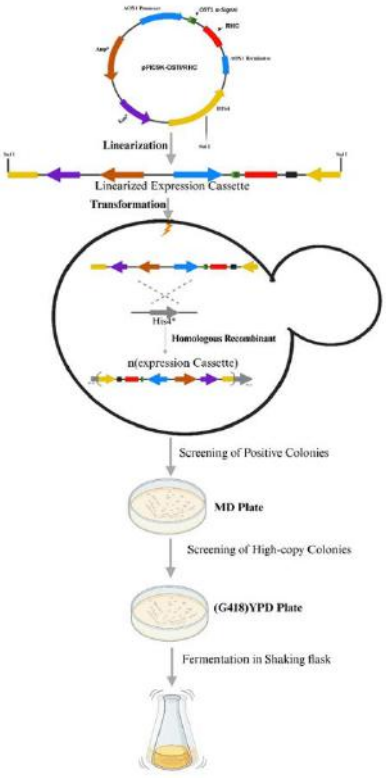

Fig.5: Overview of high-copy plasmid construction process.

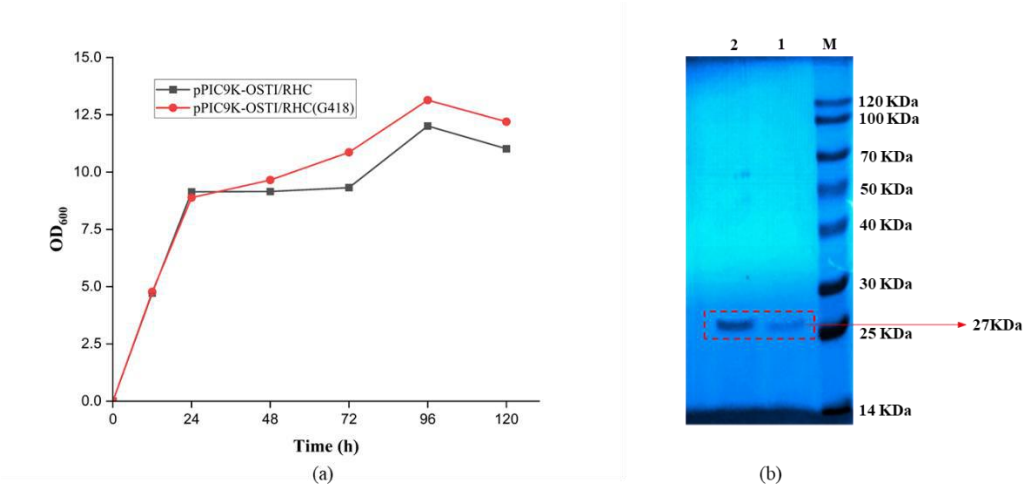

Figure 6: Growth analysis of pPIC9K-OSTI/RHC with and without G418 antibiotic. (a); SDS-PAGE analysis: Lane M (Protein Marker Blue Plus II), Lane 1(pPIC9K-OSTI/RHC without G418), Lane 2 (pPIC9K-OSTI/RHC with G418). (b)

3.4 Expression Media Optimization

After constructing the high-copy transformants with the novel plasmid pPIC9K-OSTI/RHC, we optimized the expression medium (BMMY) by testing six different pH ranges. The OD_{600} values were plotted against time (Figure 7a), and the results indicated that the pPIC9K-OSTI/RHC plasmid exhibited the highest OD_{600} value, 13.5, at pH 6, suggesting optimal growth at this pH. Figure 7b illustrates pH variations over time, showing a decreasing trend, which indicates an increase in protein production[26]. To validate these results, we performed SDS-PAGE analysis, which revealed the highest band intensity for the pPIC9K-OSTI/RHC plasmid at pH 6 (Figure 8). Additionally, protein yield measurements after fermentation in a shaker flask confirmed that the highest yield, 0.35 g/L (Figure 9), was obtained at pH 6. These findings strongly support our conclusion that pH 6 is the optimal condition for RHC protein expression using the pPIC9K-OSTI/RHC plasmid.

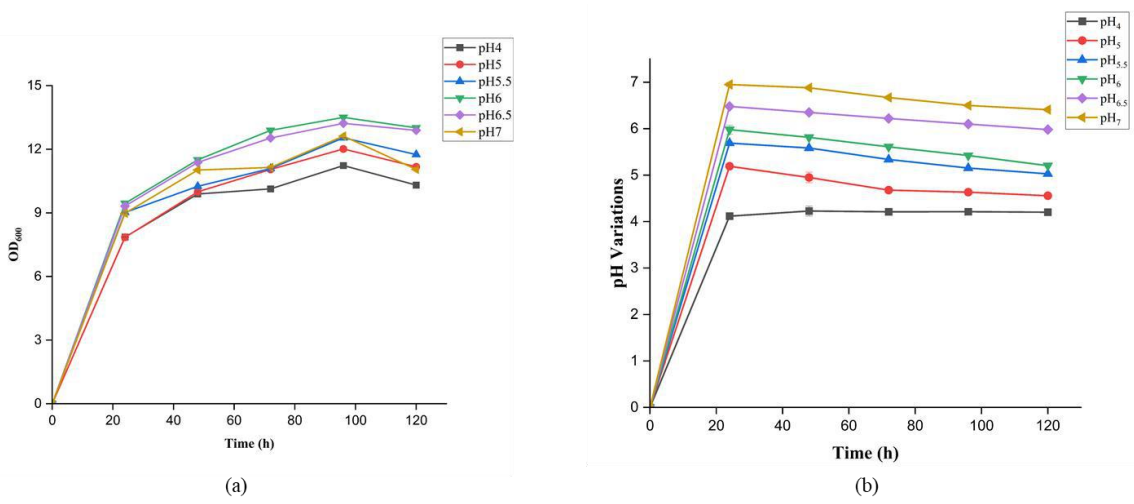

Figure 7: Fermentation media optimization at different pH levels: Analysis of the growth of host strain GS115-pPIC9K-OSTI/RHC at pH 4,5,5.5,6. (a); pH variation observation in fermentation media. (b)

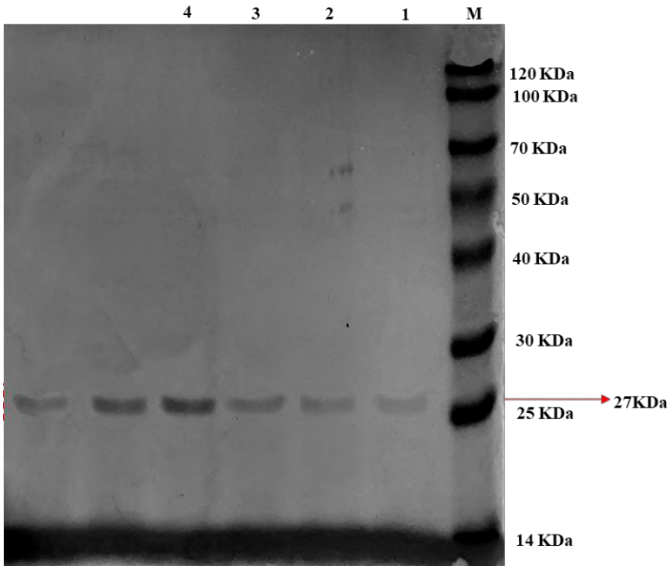

Fig.8: SDS-PAGE analysis of the supernatant. Lane M (Protein Marker Blue Plus II), Lane 1,2,3,4,5and 6 are respectively pH_4, pH_5, $pH_{5.5}$, pH_6, $pH_{6.5}$, pH_7.

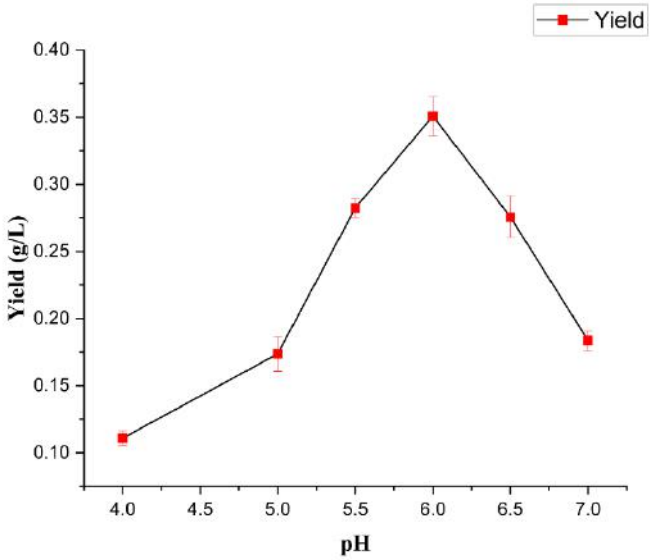

Fig.9: Protein yield for pPIC9K-OSTI/RHC plasmid at various pH.

IV. CONCLUSION

This study successfully developed a high-yield recombinant human-like collagen (RHC) production system using *P. pastoris* GS115 with a novel plasmid construct, pPIC9K-OSTI/RHC. The incorporation of the OSTI signal peptide significantly enhanced the secretory efficiency of RHC, optimizing its expression in yeast cells. We identified pH 6 as the optimal condition for recombinant protein expression, resulting in a remarkable protein yield of 0.35 g/L. The findings confirm that the use of the pPIC9K-OSTI/RHC plasmid, along with the optimized fermentation conditions, provides a cost-effective, scalable, and sustainable approach to recombinant collagen production. This method holds great potential for applications in tissue engineering, wound healing, and other biomedical fields, offering a viable alternative to traditional methods that rely on animal-based collagen extraction. The results presented here also contribute to the growing body of knowledge on the optimization of protein expression in *P. pastoris*, providing a foundation for further advancements in recombinant protein production.

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