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Research article

Optimizing Primary Isolation of Recombinant HPV L1 Protein Type 58 from *Hansenula polymorpha*

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Abstract

Human Papillomavirus (HPV) infection is a primary cause of cervical cancer. While HPV types 16 and 18 are globally recognized, HPV 58 is the predominant genotype contributing to cervical cancer incidence in Thailand. Effective prevention is offered by virus-like particle (VLP) vaccines from recombinant L1 capsid protein. However, the high cost of imported vaccines limits their affordability in Thailand, highlighting the need for domestic production to enhance accessibility and self-sustainability. Despite efficient HPV L1 protein production in large-scale bioreactors, a scalable and robust purification process remains challenging, particularly when the protein is expressed in *Hansenula polymorpha*. This study focused on optimizing the initial isolation of recombinant HPV58 L1 protein from *Hansenula polymorpha*. Cell disruption was optimized using a high-pressure homogenizer at 1000 bars. The highest L1 protein yield (76.16 µg/mL) was achieved after five homogenization passes. Agitation of the homogenate for 6 h further enhanced the L1 protein yield to 165.67 µg/mL by solubilizing protein trapped within organelles or cell debris. Protein precipitation for L1 isolation was compared using ammonium sulfate (40-50% saturation), PEG6000 (10-25% v/v), and PEG8000 (10-25% v/v). Precipitation with 45% saturated ammonium sulfate at 4°C yielded the highest specific L1 protein (45.9 µg/mL) with fewer impurities. The L1 protein recovery rate ranged from 44% to 57%. This primary isolation provides a crude L1 protein extract for further downstream purification using methods such as chromatography. The purified L1 protein can self-assemble into VLPs and can be formulated into an HPV vaccine. This work supports scalable downstream processes for industrial HPV vaccine production.

Keywords: human papillomavirus; *Hansenula polymorpha*; virus-like particles; protein precipitation; downstream process; L1 protein

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1. Introduction

Cervical cancer ranks as the fourth most common cancer among women globally, with the number of new cases increasing each year. HPV is the etiologic agent for the majority of cervical cancer cases. Both women and men are at risk of HPV infection during their lifetimes, and reinfection is possible. Approximately 90% of cervical cancer cases and related deaths occur in low- and middle-income countries. In Thailand, an estimated 9,158 women are diagnosed with cervical cancer annually, and 4,705 women die from the disease (Bruni et al., 2023). According to the World Health Organization (World Health Organization, 2025), around 300 million women currently have an HPV infection.

HPV 16 and 18 are globally the most prevalent high-risk subtypes for cervical cancer, while HPV 58 is also a significant concern in Thailand. It was among the top 5 high-risk subtypes in the country from 1989 to 2020 (Phansri et al., 2022). Studies have shown relatively higher frequencies of HPV 58 in Northern Thailand (Siriaunkgul et al., 2008), and it was the second most prevalent type among teenage girls in the Northeast (Vongpunsawad et al., 2023). Furthermore, HPV 58 prevalence was also relatively high in invasive squamous cell carcinoma among Thai women (Kietpeerakool et al., 2015). Therefore, it was anticipated that the development of HPV 58 in HPV vaccines, alongside HPV 16 and 18, could significantly improve cervical cancer prevention rates in Thailand (Suthipintawong et al., 2011).

HPV vaccines are particularly effective for prevention, especially when administered during adolescence (Kamolratanakul & Pitisuttithum, 2021). Three commercial HPV vaccines have been approved for use: Gardasil®, Gardasil 9® (Merck & Co., Inc., USA), and Cervarix™ (GlaxoSmithKline, UK) (Palefsky et al., 2011). Gardasil® protects against 4 HPV types (6, 11, 16, 18), while Gardasil 9® protects against 9 (including the original 4 plus 31, 33, 45, 52, and 58). Cervarix protects against 2 different HPV types (16 and 18) (Braaten & Laufer, 2008). Although these vaccines are effective, their high price makes them difficult to access in Thailand and other similar countries. Local production of HPV vaccines in Thailand could improve accessibility, enhance public health outcomes, and promote self-sufficiency.

The L1 protein is the main part of the HPV capsid and a key component in vaccines. Several HPV vaccines use recombinant DNA technology to produce purified L1 proteins that self-assemble into VLPs (Yazdani et al., 2020). These vaccines have demonstrated high efficacy in preventing HPV infections and cervical cancer (Ault, 2007). Beyond the major capsid protein L1, the minor capsid protein L2 has also been explored for the development of single-dose HPV vaccines. While L2 offers broad protection against multiple HPV subtypes, it lacks the intrinsic ability to self-assemble into VLPs, necessitating its conjugation with VLPs derived from bacteriophages (Shao et al., 2021). More recently, mRNA vaccines for HPV have emerged as a novel candidate vaccine platform. However, their long-term efficacy and safety profiles are not yet as extensively established as those of L1 VLP vaccines (Movahed et al., 2024).

Yeast expression systems are highly favored for VLP production due to their established safety, facile genetic manipulation, and amenability to cost-effective, large-scale manufacturing. Notably, HPV VLPs have been successfully produced in various yeast hosts, including *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Hansenula polymorpha* (Brachelente et al., 2023). Of these prominent yeast platforms, *Hansenula polymorpha* is an attractive choice for HPV VLP production, offering several advantages. It exhibits thermotolerance, growing at temperatures between 30 and 50°C. Its metabolic versatility allows it to utilize methanol as a primary carbon source, in addition to glycerol,

glucose, xylose, and cellobiose. The presence of widely utilized methanol-inducible promoters, such as formate dehydrogenase (FMD) and methanol oxidase (MOX), enables efficient recombinant protein production in this host (Manfrão-Netto et al., 2019).

The yeast strain *Hansenula polymorpha* has been genetically engineered to produce recombinant L1 protein for HPV types 6, 11, and 52. This was achieved by introducing DNA sequences encoding the respective HPV L1 capsid proteins into the yeast genome under the control of specific promoters. These engineered strains yield high amounts of recombinant L1 protein. This protein maintains the antigenic and immunogenic properties of the natural antigen (Liu et al., 2015; Yu et al., 2018). Similarly, *Hansenula polymorpha* strains capable of producing the HPV58 L1 protein have been successfully developed (Boontawon, 2017). However, effective purification methods for downstream processing remain a significant challenge. Primary isolation is a critical first step for a successful downstream process.

Cell disruption is the initial step in releasing the L1 protein produced within cells. High-pressure homogenization is effective for breaking yeast cells by utilizing turbulence, elongational and shear stresses, and cavitation to fragment the cell walls. This method has been successfully applied to various VLP-expressing yeast strains. These include *Saccharomyces cerevisiae* (Kee et al., 2010), *Pichia pastoris* (Lünsdorf et al., 2011), and *Hansenula polymorpha* (Huang et al., 2007). Optimizing homogenization parameters can improve the protein yield and minimize operational costs. These parameters include pressure, temperature, number of cycles, and buffer solution ratio (Ekpeni et al., 2015).

Protein precipitation is a crucial step in the isolation of the target L1 protein from the cell homogenate. This helps reduce the operational volume for cost-efficient large-scale production. Common precipitants for VLP recovery are ammonium sulfate (Kim et al., 2009; Firdaus et al., 2023) and polyethylene glycol (PEG) (Zahid et al., 2015). However, optimizing this process is essential to efficiently recover HPV L1 protein from recombinant *Hansenula polymorpha*.

This study aims to optimize the primary isolation of HPV58 L1 protein from *Hansenula polymorpha*. The process includes cell disruption, extraction of L1 protein from cell debris and organelles, and protein precipitation to maximize the yield of L1 protein. The crude L1 protein is further purified using tangential flow filtration and chromatography. The goal is to prepare purified L1 protein for vaccine formulation.

2. Materials and Methods

2.1 Cell strain and production

Recombinant *Hansenula polymorpha* strain HPV58 employed in this research was constructed by Boontawon (2017) based on *Hansenula polymorpha* NCYC495 (SH4330) ura3 (Ura-) (provided by Yeast Genetic Resource Center (YGRC), Osaka University, Japan). A chromosome integrated plasmid pTB9 (HPV 58 L1) was constructed based on a backbone of plasmid pUC57 (derivative of pUC19) and was harbored in the strain. The cells were streaked on SC-Ura agar plates and incubated at 30°C for 3 to 5 days before being inoculated into 100 mL of SC-Ura broth. The broth culture was incubated at 30°C and 250 rpm for 48 h. After 48 h, the inoculum was mixed with 350 mL of Syn6 medium using glycerol as the carbon source. This mixture was aliquoted into 4 flasks (100 mL each) and incubated under similar conditions for an additional 48 h. The 400 mL culture was then scaled up by mixing with 3,600 mL of Syn6 medium in a bioreactor. The cultivation conditions in the bioreactor were maintained at 30°C, 1000 rpm, 1 vvm air flow rate, and

pH 5.5. After 32 h, methanol induction (500 g/L) was initiated using DOT stat control, and the cultivation was continued under the same conditions. After 72 h of total cultivation, the cells were harvested by centrifugation. The harvested cells were stored at -80°C before downstream processing.

2.2 Cell disruption by high-pressure homogenizer

Frozen *Hansenula polymorpha* HPV58 cells were re-suspended in the breaking buffer (PBS containing 5 mM EDTA and 0.1% v/v Tween20) to a final volume of 500 mL of 15% (w/v) of solid content. The cell suspension was then loaded into the high-pressure homogenizer (Gea Panda 2000 Plus). The pressure was operated at 1000 bars. After each pass, the samples were collected and centrifuged at 14000 rpm at 4°C for 10 min for total protein and L1 protein analysis. Optical density at 660 nm (OD_{660}) of cell homogenate was also measured to confirm cell disruption.

2.3 Agitation of cell homogenate

The cell homogenate was stirred at 260 rpm, 4°C following cell disruption. Samples were collected at 0, 3, 6, 9, 12, 24, and 48 h after starting agitation for total protein and L1 protein analysis. Prior to analysis, the samples were centrifuged as described previously to remove cell debris.

2.4 Protein precipitation by ammonium sulfate and polyethylene glycol (PEG)

After agitation and cell debris removal, the crude protein was precipitated using ammonium sulfate powder (40%, 45%, and 50% saturation), PEG6000 solution (10-25% v/v), and PEG8000 solution (10-25% v/v). Ammonium sulfate concentrations were chosen based on previous studies of L1 protein precipitation from *Hansenula polymorpha* (Firdaus et al., 2023). The selected ranges for PEG concentration were informed by precipitation studies of the recombinant hepatitis B surface antigen (HBsAg), a model VLP expressed in *Saccharomyces cerevisiae* (Kee, 2009). The suspension was continuously stirred at 4°C for 1 h after ammonium sulfate had been completely dissolved. Protein pellets were then separated by centrifugation at 12000 rpm 4°C for 10 min. The pellets were re-suspended in PBS-EDTA buffer (pH 7.4) and then dialyzed using centrifugal filters with 30,000 MWCO (Millipore Amicon® Ultra-15) for five exchanges of the sample volume. The supernatants were also dialyzed using the same method. Both pellets and supernatants were analyzed for total protein (Bradford assay), L1 protein (indirect ELISA assay), and by SDS-PAGE.

2.5 Sample analysis

2.5.1 Total protein determination

The samples were mixed with Bradford protein assay reagent (Bio-Rad Laboratories, USA) and incubated for 5 min before the absorbance at 595 nm was read. A standard curve was constructed using bovine serum albumin.

2.5.2 Indirect ELISA for HPV L1 protein determination

The indirect ELISA was performed with minor modifications to the method described by Kohl & Ascoli (2017). The samples and freshly prepared standard HPV L1 protein were diluted to the appropriate concentrations in 1X PBS buffer. Subsequently, 100 µL of the diluted protein was added to each well of an ELISA plate and incubated overnight at 4°C, with the plate wrapped in parafilm. To assess nonspecific antibody reactivity, control wells were coated with protein extract prepared from recombinant yeast harboring an empty vector, grown under identical conditions. Following incubation, the plate was washed three times with 300 µL per well of washing buffer (1X PBS with 0.05% Tween 20, referred to as PBS-T buffer). Each well was then blocked with 200 µL of blocking buffer (0.5% w/v BSA in 1X PBS-T) and incubated at 37°C for 1 h. The plate was washed again as described above. A 1:2000 dilution of mouse anti-L1 HPV16 monoclonal antibody (CAMVIR-I) in blocking buffer was prepared, and 100 µL was added to each well. The plate was incubated at 37°C for 1 h and washed five times using PBS-T buffer. Horse anti-mouse IgG conjugated with HRP was diluted 1:3000 in blocking buffer, and 100 µL was added to each well. After incubation, the plate was washed five times as before. To develop the color, 100 µL of room-temperature TMB solution was added to each well, and the plate was incubated at room temperature in the dark for 20 min. The reaction was stopped by adding 100 µL of 1 M H_2SO_4, causing the color to change from blue to yellow upon acidification. The absorbance at 450 nm was measured and compared with a standard curve generated using the HPV L1 protein.

2.5.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein electrophoresis was conducted using a Mini Protein II Cell Electrophoresis Set or Mini-Slab Size Electrophoresis System. The protein sample was mixed with the sample buffer containing dye before boiling at 100°C for 10 min. After cooling down, 10 µg of the protein mixture was loaded and run at a constant voltage of 120 V in a 1x running SDS buffer until the loading dye migrates to the bottom of the gel, which typically required approximately 2.5 h. The molecular weights of the proteins were compared to the Precision Plus Protein™ molecular weight marker. After electrophoresis, the gel was stained with Coomassie Blue R-250 solution to visualize the protein bands.

2.5.4 Statistical analysis

All experiments were conducted in triplicate. The statistical analyses were performed by a SPSS package using a one-way analysis of variance (ANOVA) at $p<0.05$. The least significant difference (LSD) was used for post hoc test at the 95% confidence level.

3. Results and Discussion

3.1 Cell disruption

Cell disruption by high-pressure homogenizer was selected for this study due to the disruption efficiency and scalability of the method. The total protein concentrations of cell homogenates after each pass are presented in Figure 1. The highest protein concentration (14.24 mg/mL) was achieved after 6 passes of homogenization. Total protein concentration after each pass was significantly different ($p<0.001$). However, no significant differences in

protein concentrations were observed between four and five passes (p = 0.373), five and six passes (p = 0.768), or four and six passes (p = 0.243), suggesting that complete cell disruption was largely achieved by the fourth pass. This finding also correlated with optical density (OD) measurements (data not shown), where OD values decreased after each pass, with the lowest OD observed at six passes.

The HPV58 L1 protein concentration after each pass was significantly different (p<0.001). Due to excessive heat generation observed after six passes, which is not an optimal condition for further production, L1 protein concentrations were quantified only for passes one to five (Figure 2). The highest L1 protein concentration (76.16 µg/mL) was observed after 5 passes of homogenization, which was significantly higher than that achieved after four passes (p = 0.002). Thus, five cycles of homogenization were determined to be optimal for cell disruption to achieve a high yield of L1 protein.

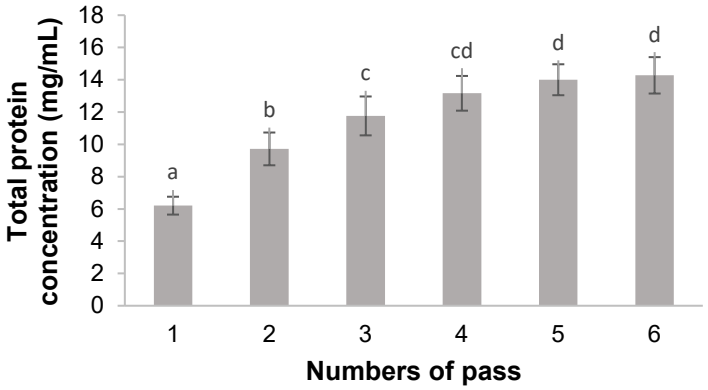

Figure 1 Concentration of total protein in the homogenate after different numbers of passes at 1000 bars. The data were obtained from three independent experiments, and values are expressed as mean±SD. Different superscripts on the bars indicate significant difference at p<0.05.

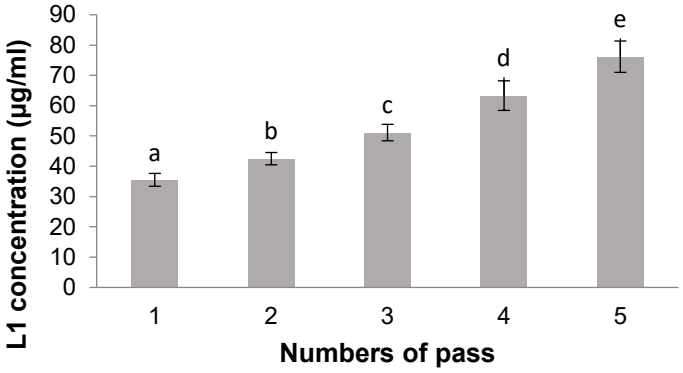

Figure 2 Concentration of HPV58 L1 protein in the homogenate after different numbers of passes at 1000 bars. The data were obtained from three independent experiments, and values are expressed as mean±SD. Different superscripts on the bars indicate significant difference at p<0.05.

High-pressure homogenization is one of the most effective methods for disrupting *Hansenula polymorpha* cells (Huang et al., 2007; Xu et al., 2014). Pressure exceeding 1000 bars and more than five passes were required for effective cell disruption (Wetzel et al., 2018, 2019). These conditions are more extreme than those typically used for other yeast species (Jiang et al., 2011; Rosmeita et al., 2023). The relatively strong cell wall of *Hansenula polymorpha*, particularly when grown in glycerol/methanol medium, contributes to its resistance to disruption compared to other yeast cell walls (Giuseppin et al., 1987).

3.2 Agitation of cell homogenate

Cell homogenate was agitated at a constant rate to facilitate the solubilization of L1 protein trapped within cell debris or organelles. The highest L1 yield (165.67 µg/mL) was observed after 6 h of agitation which was significantly higher than that achieved after 3 h of agitation ($p<0.001$) (Figure 3). The presence of detergents such as Tween20 in the breaking buffer can further enhance protein extraction from yeast cells by weakening cell wall structures and aiding in solubilization during agitation (Zhang et al., 2011).

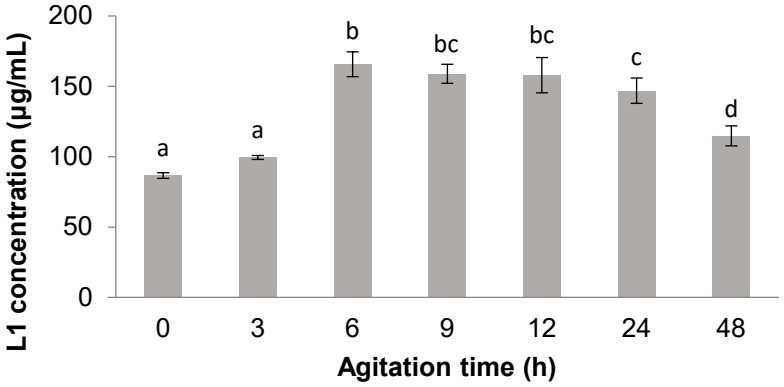

Figure 3. Effects of agitation time on HPV58 L1 protein in the homogenate. The data were obtained from three independent experiments, and values are expressed as mean±SD. Different superscripts on the bars indicate significant difference at $p<0.05$.

The yield of L1 protein did not improve with agitation beyond 6 h. The results also indicated that extended agitation, particularly up to 48 h, significantly reduced the L1 protein yield ($p<0.001$), likely due to degradation by host cell proteases. Therefore, a six-hour agitation period was chosen to balance a shorter process time with the maintenance of L1 protein yield.

3.3 Protein precipitation

3.3.1 Ammonium sulfate precipitation

The effect of ammonium sulfate concentration on protein precipitation was examined (Table 1). The highest L1 protein concentration (390.92 µg/mL) was obtained from the pellet precipitated by 45% saturation of ammonium sulfate. This result was consistent with the corresponding supernatants, which showed low L1 concentrations in samples from

45% and 50% saturation. In contrast, at 40% saturation, less L1 protein was precipitated, resulting in higher L1 concentrations in the supernatant. These findings align with previous studies on the recovery of HPV16 L1 protein from *Saccharomyces cerevisiae* cell lysate (Park et al., 2008).

Table 1. Effects of ammonium sulfate concentration on recovery of HPV58 L1 protein in pellet and supernatants

Ammonium Sulfate Concentration (%Saturation)	L1 Concentration (µg/mL)		Specific L1 (µg/mg Total Protein)	
	Pellet	Supernatant	Pellet	Supernatant
40%	193.56 ± 4.28^{a}	11.00 ± 1.42^{a}	6.47 ± 0.14^{a}	2.06 + 0.27^{a}
45%	390.92 ± 12.78^{b}	1.54 ± 0.65^{b}	9.99 ± 0.33^{b}	0.36 + 0.15^{b}
50%	317.99 ± 12.20^{c}	1.33 ± 0.28^{b}	7.79 ± 0.30^{c}	0.37 + 0.08^{b}

Different superscripts within a column indicate significant difference at $p < 0.05$.

The specific L1 protein content from the 45% saturation ammonium sulfate precipitate was significantly higher than that from the 40% ($p<0.001$) and 50% ($p<0.001$) saturation precipitates. Therefore, precipitation at 45% saturation ammonium sulfate likely provided better selectivity for the L1 protein.

3.3.2 Polyethylene glycol (PEG) precipitation

PEG6000 and PEG8000 at various concentrations were screened for L1 protein precipitation on a small scale (Table 2). The L1 protein precipitates obtained with 25% v/v PEG6000 were significantly higher than those achieved with 10% v/v ($p = 0.008$) and 20% v/v ($p = 0.003$) PEG6000. In contrast, no significant differences in L1 protein concentrations were observed among pellets precipitated using different concentrations of PEG8000 ($p = 0.434$). Interestingly, PEG6000 and PEG8000 concentrations were found to influence the yield of Hepatitis B VLP from *Saccharomyces cerevisiae* cell lysate (Tsoka et al., 2000). However, the precipitates from 10% and 15% v/v of both PEG6000 and PEG8000 exhibited high specific L1 protein values. Higher PEG concentrations may promote co-precipitation of impurity proteins, thereby reducing the ratio of L1 protein to total protein. Based on these findings, 15% v/v PEG8000 was selected for further study due to its ability to achieve the highest specific L1 protein yield.

Table 2. Effects of PEG concentration on recovery of HPV58 L1 protein in pellets

PEG (%v/v)	L1 Concentration (µg/mL)		Specific L1 (µg/mg Total Protein)	
	PEG6000	PEG8000	PEG6000	PEG8000
10%	10.57 ± 0.45^{ab}	10.01 ± 0.32^{a}	20.13 ± 0.86^{a}	18.35 + 0.58^{a}
15%	11.53 ± 0.61^{ac}	10.37 ± 0.40^{a}	20.06 ± 1.06^{a}	21.78 + 0.85^{b}
20%	10.25 ± 0.27^{b}	10.50 ± 0.66^{a}	16.35 ± 0.42^{b}	16.06 + 1.00^{c}
25%	12.30 ± 0.89^{c}	10.90 ± 0.95^{a}	19.19 ± 1.39^{a}	16.46 ± 1.44^{ac}

Different superscripts within a column indicate significant difference at $p < 0.05$.

3.3.3 L1 protein precipitation between ammonium sulfate and PEG

The precipitation of L1 protein using 45% saturation ammonium sulfate achieved a higher yield (45.9 µg/mL) compared to 15% v/v PEG8000 (Figure 4). A significantly higher specific L1 protein was also observed with 45% saturation ammonium sulfate ($p = 0.021$), suggesting that this method resulted in fewer impurity proteins in the crude L1 pellet. This finding was consistent with the SDS-PAGE results shown in Figure 5. The protein band at 56 kDa, which was expected to be the L1 protein, was observed in protein precipitates from ammonium sulfate and PEG. In the protein precipitates obtained with PEG, a prominent impurity protein band at 75 kDa was observed, along with other bands around 37 kDa. This suggests that small and large impurity proteins were precipitated with L1 protein when using PEG. While PEG was effective for isolating hepatitis B VLP from *Saccharomyces cerevisiae* (Tsoka et al., 2000) and *Hansenula polymorpha* (Huang et al., 2007; Wetzel et al., 2018) cell homogenates, its effectiveness was not clearly demonstrated for HPV58 L1 protein isolation from *Hansenula polymorpha*. Conversely, ammonium sulfate at 45% saturation showed strong potential for L1 protein isolation.

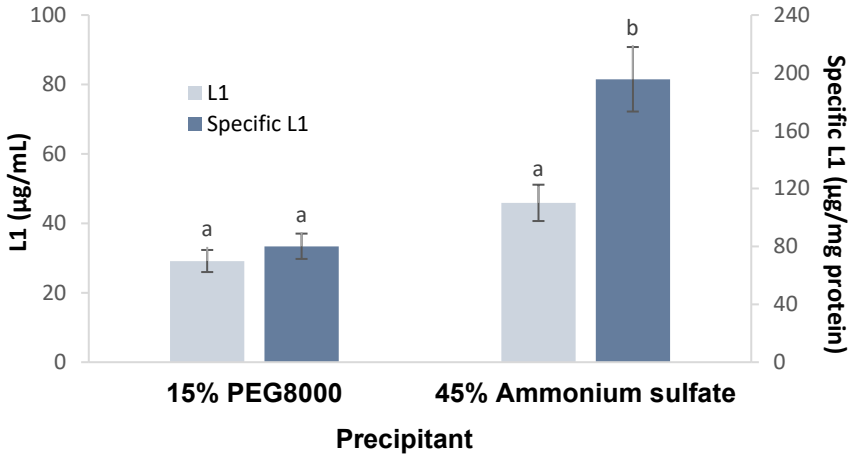

Figure 4. Precipitation of HPV58 L1 protein using different precipitants. Different superscripts within similar color bars indicate significant difference at $p<0.05$.

The recovery yield of HPV58 L1 protein in this study ranged from 44% to 57%. This recovery rate is comparable to those reported for L1 proteins from other HPV subtypes. For instance, L1 protein recoveries from the insect-baculovirus expression system for HPV16, HPV18, HPV6, and HPV11 were reported as 72%, 47%, 66%, and 64%, respectively (Aghajani et al., 2024). However, anion exchange chromatography for direct L1 protein isolation from cell lysate was utilized. Similarly, a 51.76% recovery of HPV52 L1 protein directly from *Hansenula polymorpha* cell lysate using cation exchange chromatography was reported by Chairunnisa et al. (2025) . While these recovery rates were comparable to our findings, the direct loading of crude cell lysate onto chromatography columns presents significant challenges, particularly for large-scale production. The high concentration of impurity proteins and large operational volumes necessitate substantial quantities of chromatography resin, leading to increased costs.

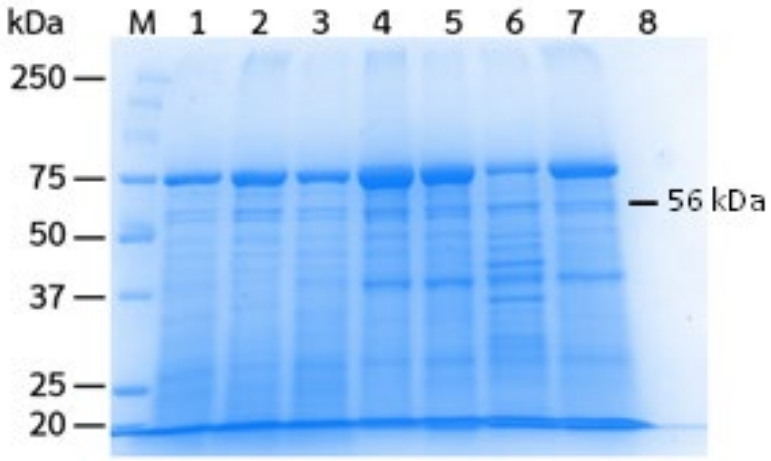

Figure 5. Resuspended and dialyzed protein precipitates of 10 µg were loaded on SDS-PAGE staining with Coomassie Brilliant blue. The molecular weight of L1 protein is around 56 kDa. Each lane represents as follows; M: Precision Plus Protein All Blue Standards Markers (Bio-Rad),1: After cell disruption, 2: Protein precipitation with 45% ammonium sulfate, 3: Protein precipitation with 0% PEG, 4: Protein precipitation with 15% PEG 6000, 5: Protein precipitation with 20% PEG 6000, 6: Protein precipitation with 15% PEG 8000, 7: Protein precipitation with 20% PEG 8000.

In contrast, considerably lower HPV52 L1 protein recoveries of 4.31% from *Hansenula polymorpha* and 5.90% from *Pichia pastoris* was reported by Firdaus et al. (2023). Although ammonium sulfate precipitation was also employed in their study, a higher saturation (50%) was used, which may have negatively impacted the recovery yield. For comparison, HPV16 L1 protein recoveries ranging from 75% to 88% were reported using 45% saturation ammonium sulfate precipitation in *Saccharomyces cerevisiae* (Park et al., 2008; Kim et al., 2009). The higher recovery rates observed in these studies are potentially attributed to differences in host cell systems, HPV subtypes, and the inclusion of protease inhibitors in the cell lysis buffer.

Primary isolation of HPV58 L1 protein from *Hansenula polymorpha* was optimized using cell disruption, cell homogenate agitation, and protein precipitation. However, there were limitations in this study. Even though the recovery yield of L1 protein was acceptable, this could be improved by reducing L1 protein degradation. Adding effective protein inhibitors and/or protein stabilizers to the buffer might reduce degradation rate and improve the yield. This study aimed at the primary isolation of HPV58 L1 protein, which required optimization in each step and focus on recovery. Thus, purification was not prioritized and the purity of HPV58 L1 protein needs to be massively improved in further study. Chromatography should be used for purification to achieve high purity of HPV58 L1 protein. Due to limitation of L1 protein purity in this study, characterization and functionality of HPV58 L1 protein would be challenging to demonstrate. However, HPV58 L1 protein expressed from *Hansenula polymorpha* could self-assemble into VLP under transmission electron microscopy (TEM) as reported by Boontawon (2017). Structure integrity and immunogenicity of HPV58 VLP was expected after isolation of L1 protein using ammonium sulfate precipitation (Kwag et al., 2012).

4. Conclusions

The process of isolating recombinant HPV58 L1 protein was investigated starting from cell disruption. *Hansenula polymorpha* cells were resuspended in breaking buffer and disrupted using a high-pressure homogenizer at 1000 bars. Homogenization for five cycles yielded the highest L1 protein concentration of 76.16 µg/mL. Subsequent agitation at 4°C for 6 h improved the L1 protein yield to 165.67 µg/mL. Protein precipitation was then studied using various precipitants, including ammonium sulfate (40-50% saturation), PEG6000 (10-25% v/v), and PEG8000 (10-25% v/v). Ammonium sulfate at 45% saturation exhibited the highest L1 protein yield (45.9 µg/mL) with fewer impurity proteins. The recovery rate of the L1 protein ranged from 44% to 57%. However, further optimization could enhance the recovery and yield. This research establishes a foundational framework for the downstream processing of recombinant HPV58 L1 protein expressed in *Hansenula polymorpha*. This work directly contributes to the development of an HPV VLP-based vaccine platform and enhances vaccine production capabilities within Thailand. The established isolation process is also broadly applicable to the downstream processing of L1 proteins from other HPV subtypes. Thereby, this facilitates the production of multivalent HPV vaccines tailored to the specific epidemiological needs of Thailand. Future investigations will focus on enhancing the purity of HPV VLPs through the employment of heparin or cation exchange chromatography. Subsequent characterization and immunogenicity studies of the purified HPV VLPs will be crucial to validate product quality and optimize the downstream process prior to vaccine formulation. As the scalability of the isolation and purification methods employed, future work will also encompass the scale-up of these downstream processes for potential industrial production. This has significant potential to foster self-sustainability in vaccine production and strengthen cervical cancer prevention efforts in Thailand.

5. Acknowledgements

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6. Authors' Contributions

N.K. and P.P. performed the experiments, with a cell production provided by T.C. T.T, C.B., and T.P. designed the experiments. N.K. and T.T. analyzed the data and wrote the manuscript with essential support from C.B. and T.P. All authors reviewed and approved the manuscript.

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7. Conflicts of Interest

There is no conflict of interest.

References

Aghajani, K., Asadollahi, A., Taghizadeh, T., Rahmati, F., Dehghani, M., & Mirhassani, R. (2024). Strain-specific differences in purification and VLP formation for a quadrivalent recombinant HPV vaccine. *Biochemical Engineering Journal*, *209*, Article 109379. https://doi.org/10.1016/j.bej.2024.109379

Ault, K. A. (2007). Effect of prophylactic human papillomavirus L1 virus-like-particle vaccine on risk of cervical intraepithelial neoplasia grade 2, grade 3, and adenocarcinoma in situ: a combined analysis of four randomised clinical trials. *The Lancet*, *369*(9576), 1861-1868. https://doi.org/10.1016/S0140-6736(07)60852-6

Boontawon, T. (2017). *Expression of the L1 major capsid protein of human papillomavirus type 52 and 58* [unpublished Master thesis]. Mahidol University.

Braaten, K. P., & Laufer, M. R. (2008). Human Papillomavirus (HPV), HPV-Related Disease, and the HPV Vaccine. *Reviews in Obstetrics and Gynecology*, 1(1), 2-10.

Brachelente, S., Galli, A., & Cervelli, T. (2023). Yeast and virus-like particles: A perfect or imperfect couple? *Applied Microbiology* *3*(3), 805-825. https://doi.org/10.3390/applmicrobiol3030056

Bruni, L., Albero, G., Serrano, B., Mena, M., Collado, J. J., Gómez, D., Muñoz, J., Bosch, F. X., & de Sanjosé, S. (2023). *Human papillomavirus and related diseases. report in the world*. https://hpvcentre.net/statistics/reports/XWX.pdf

Chairunnisa, S., Mustopa, A. Z., Bela, B., Arifah, R. K., Umami, R. N., Firdaus, M. E. R., Ekawati, N., Irawan, H., Irawan, S., Nurfatwa, M., Hertati, A., Swasthikawati, S., Novianti, E., Kusumawati, A., & Darusman, H. S. (2025). Expression, purification, and immunogenicity study of human papillomavirus type 52 virus-like particles produced in Hansenula polymorpha. *Biologicals*, *90*, Article 101831. https://doi.org/10.1016/j.biologicals.2025.101831

Ekpeni, L. E. N., Benyounis, K. Y., Nkem-Ekpeni, F. F., Stokes, J., & Olabi, A. G. (2015). Underlying factors to consider in improving energy yield from biomass source through yeast use on high-pressure homogenizer (hph). *Energy*, *81*, 74-83. https://doi.org/10.1016/j.energy.2014.11.038

Firdaus, M. E. R., Mustopa, A. Z., Ekawati, N., Chairunnisa, S., Arifah, R. K., Hertati, A., Irawan, S., Prastyowati, A., Kusumawati, A., & Nurfatwa, M. (2023). Optimization, characterization, comparison of self-assembly VLP of capsid protein L1 in yeast and reverse vaccinology design against human papillomavirus type 52. *Journal of Genetic Engineering and Biotechnology*, *21*(1), Article 68. https://doi.org/10.1186/s43141-023-00514-9

Giuseppin, M. L. F., van Eijk, H. M. J., Hellendoorn, M., & van Almkerk, J. W. (1987). Cell wall strength of *Hansenula polymorpha* in continuous cultures in relation to the recovery of methanol oxidase (MOX). *Applied Microbiology and Biotechnology*, *27*(1), 31-36. https://doi.org/10.1007/BF00257250

Huang, Y., Bi, J., Zhang, Y., Zhou, W., Li, Y., Zhao, L., & Su, Z. (2007). A highly efficient integrated chromatographic procedure for the purification of recombinant hepatitis B surface antigen from *Hansenula polymorpha*. *Protein Expression and Purification*, *56*(2), 301-310. https://doi.org/10.1016/j.pep.2007.08.009

Jiang, Z., Tong, G., Cai, B., Xu, Y., & Lou, J. (2011). Purification and immunogenicity study of human papillomavirus 58 virus-like particles expressed in *Pichia pastoris*. *Protein Expression and Purification*, *80*(2), 203-210. https://doi.org/10.1016/j.pep.2011.07.009

Kamolratanakul, S., & Pitisuttithum, P. (2021). Human papillomavirus vaccine efficacy and effectiveness against cancer. *Vaccines*, *9*(12), Article 1413. https://doi.org/10.3390/vaccines9121413

Kee, G. S. (2009). *A rational approach to the development of future generation processes for lipoprotein VLP vaccine candidates* [Ph.D. thesis, University College London]. https://discovery.ucl.ac.uk/id/eprint/18768/1/18768.pdf

Kee, G. S., Jin, J., Balasundaram, B., Bracewell, D. G., Pujar, N. S., & Titchener-Hooker, N. J. (2010). Exploiting the intracellular compartmentalization characteristics of the *S. cerevisiae* host cell for enhancing primary purification of lipid-envelope virus-like particles. *Biotechnology Progress*, *26*(1), 26-33. https://doi.org/10.1002/btpr.307

Kietpeerakool, C., Kleebkaow, P., & Srisomboon, J. (2015). Human papillomavirus genotype distribution among thai women with high-grade cervical intraepithelial lesions and invasive cervical cancer: a literature review. *Asian Pacific Journal of Cancer Prevention*, *16*(13), 5153-5158.

Kim, H. J., Lim, S. J., Kim, J. Y., Kim, S. Y., & Kim, H. J. (2009). A method for removing contaminating protein during purification of human papillomavirus type 18 L1 protein from Saccharomyces cerevisiae. *Archives of Pharmacal Research*, *32*(12), 1759-1766. https://doi.org/10.1007/s12272-009-2214-x

Kohl, T. O., & Ascoli, C. A. (2017). Immunometric double-antibody sandwich enzyme-linked immunosorbent assay. *Cold Spring Harbor Protocols*, *2017*(6), Article pdb.prot093724. https://doi.org/10.1101/pdb.prot093724

Kwag, H.-L., Kim, H. J., Chang, D. Y., & Kim, H.-J. (2012). The production and immunogenicity of human papillomavirus type 58 virus-like particles produced in *Saccharomyces cerevisiae*. *Journal of Microbiology*, *50*(5), 813-820. https://doi.org/10.1007/s12275-012-2292-1

Liu, C., Yao, Y., Yang, X., Bai, H., Huang, W., Xia, Y., & Ma, Y. (2015). Production of recombinant human papillomavirus type 52 L1 protein in Hansenula polymorpha formed virus-like particles. *Journal of Microbiology and Biotechnology*, *25*(6), 936-940. https://doi.org/10.4014/jmb.1412.12027

Lünsdorf, H., Gurramkonda, C., Adnan, A., Khanna, N., & Rinas, U. (2011). Virus-like particle production with yeast: ultrastructural and immunocytochemical insights into *Pichia pastoris* producing high levels of the Hepatitis B surface antigen. *Microbial Cell Factories*, *10*(1), Article 48. https://doi.org/10.1186/1475-2859-10-48

Manfrão-Netto, C., H. C., Gomes, A. M. V., & Parachin, N. S. (2019). Advances in using *Hansenula polymorpha* as chassis for recombinant protein production. *Frontiers in Bioengineering and Biotechnology*, *7*, Article 94. https://doi.org/10.3389/fbioe.2019.00094

Movahed, F., Darzi, S., Mahdavi, P., Salih Mahdi, M., Qutaiba B. Allela, O., Naji Sameer, H., Adil, M., Zarkhah, H., Yasamineh, S., & Gholizadeh, O. (2024). The potential use of therapeutics and prophylactic mRNA vaccines in human papillomavirus (HPV). *Virology Journal, 21*(1), Article 124. https://doi.org/10.1186/S12985-024-02397-9

Palefsky, J. M., Giuliano, A. R., Goldstone, S., Moreira, E. D. Jr., Aranda, C., Jessen, H., Hillman, R., Ferris, D., Coutlee, F., Stoler, M. H., Marshall, J. B., Radley, D., Vuocolo, S., Haupt, R. M., Guris, D., & Garner, E. I. O. (2011). HPV vaccine against anal HPV infection and anal intraepithelial neoplasia. *The New England Journal of Medicine*, *365*(17), 1576-1585. https://doi.org/10.1056/NEJMoa1010971

Park, M. A., Kim, H. J., & Kim, H. J. (2008). Optimum conditions for production and purification of human papillomavirus type 16 L1 protein from *Saccharomyces cerevisiae*. *Protein Expression and Purification*, *59*(1), 175-181. https://doi.org/10.1016/j.pep.2008.01.021

Phansri, T., Boonmark, K., & Ngamkham, J. (2022). Retrospective study of human papilloma virus infection in Thailand: A Systematic Review. *Thai Cancer Journal*, *42*(1), 10-29.

Rosmeita, C. N., Budiarti, S., Mustopa, A. Z., Novianti, E., Swasthikawati, S., Chairunnisa, S., Hertati, A., Nurfatwa, M., Ekawati, N., & Hasan, N. (2023). Expression, purification, and characterization of self-assembly virus-like particles of capsid protein L1 HPV 52 in *Pichia pastoris* GS115. *Journal of Genetic Engineering and Biotechnology*, *21*(1), Article 126. https://doi.org/10.1186/s43141-023-00571-0

Shao, S., Ortega-Rivera, O. A., Ray, S., Pokorski, J. K., & Steinmetz, N. F. (2021). A scalable manufacturing approach to single dose vaccination against HPV. *Vaccines*, *9*(1), Article 66. https://doi.org/10.3390/vaccines9010066

Siriaunkgul, S., Suwiwat, S., Settakorn, J., Khunamornpong, S., Tungsinmunkong, K., Boonthum, A., Chaisuksunt, V., Lekawanvijit, S., Srisomboon, J., & Thorner, P. S. (2008). HPV genotyping in cervical cancer in Northern Thailand: Adapting the linear array HPV assay for use on paraffin-embedded tissue. *Gynecologic Oncology*, *108*(3), 555-560. https://doi.org/10.1016/j.ygyno.2007.11.016

Suthipintawong, C., Siriaunkgul, S., Tungsinmunkong, K., Pientong, C., Ekalaksananan, T., Karalak, A., Kleebkaow, P., Vinyuvat, S., Triratanachat, S., & Khunamornpong, S. (2011). Human papilloma virus prevalence, genotype distribution, and pattern of infection in Thai women. *Asian Pacific Journal of Cancer Prevention*, *12*(4), 853-856.

Tsoka, S., Ciniawskyj, O. C., Thomas, O. R. T., Titchener-Hooker, N. J., & Hoare, M. (2000). Selective flocculation and precipitation for the improvement of virus-like particle recovery from yeast homogenate. *Biotechnology Progress*, *16*(4), 661-667. https://doi.org/10.1021/bp0000407

Vongpunsawad, S., Rhee, C., Nilyanimit, P., Poudyal, N., Jiamsiri, S., Ahn, H. S., Lee, J., Seo, H. W., Klinsupa, W., Park, S., Premsri, N., Namwat, C., Silaporn, P., Excler, J. L., Kim, D. R., Markowitz, L. E., Unger, E. R., Rerks-Ngarm, S., Lynch, J., & Poovorawan, Y. (2023). Prevalence of HPV infection among Thai schoolgirls in the north-eastern provinces in 2018: implications for HPV immunization policy. *IJID Regions*, *7*, 110-115. https://doi.org/10.1016/J.IJREGI.2023.02.011

Wetzel, D., Barbian, A., Jenzelewski, V., Schembecker, G., Merz, J., & Piontek, M. (2019). Bioprocess optimization for purification of chimeric VLP displaying BVDV E2 antigens produced in yeast *Hansenula polymorpha*. *Journal of Biotechnology*, *306*, 203-212. https://doi.org/10.1016/j.jbiotec.2019.10.008

Wetzel, D., Rolf, T., Suckow, M., Kranz, A., Barbian, A., Chan, J.-A., Leitsch, J., Weniger, M., Jenzelewski, V., Kouskousis, B., Palmer, C., Beeson, J. G., Schembecker, G., Merz, J., & Piontek, M. (2018). Establishment of a yeast-based VLP platform for antigen presentation. *Microbial Cell Factories*, *17*(1), Article 17. https://doi.org/10.1186/s12934-018-0868-0

World Health Organization. (2025, September 10). Sexually transmitted infections (STIs). https://www.who.int/news-room/fact-sheets/detail/sexually-transmitted-infections-(stis)

Xu, X., Ren, S., Chen, X., Ge, J., Xu, Z., Huang, H., Sun, H., Gu, Y., Zhou, T., Li, J., & Xu, H. (2014). Generation of hepatitis B virus PreS2-S antigen in *Hansenula polymorpha*. *Virologica Sinica*, 29(6), 403-409. https://doi.org/10.1007/s12250-014-3508-9

Yazdani, Z., Rafiei, A., Valadan, R., Ashrafi, H., Pasandi, M. S., & Kardan, M. (2020). Designing a potent L1 protein-based HPV peptide vaccine: A bioinformatics approach. *Computational Biology and Chemistry*, *85*, Article 107209. https://doi.org/10.1016/j.compbiolchem.2020.107209

Yu, Y., Guo, J., Li, D., Liu, Y., Yu, Y., & Wang, L. (2018). Development of a human papillomavirus type 6/11 vaccine candidate for the prevention of *Condyloma acuminatum*. *Vaccine*, *36*(32), 4927-4934. https://doi.org/10.1016/j.vaccine.2018.06.041

Zahid, M., Lünsdorf, H., & Rinas, U. (2015). Assessing stability and assembly of the hepatitis B surface antigen into virus-like particles during down-stream processing. *Vaccine*, *33*(31), 3739-3745. https://doi.org/10.1016/j.vaccine.2015.05.066

Zhang, T., Lei, J., Yang, H., Xu, K., Wang, R., & Zhang, Z. (2011). An improved method for whole protein extraction from yeast *Saccharomyces cerevisiae*. *Yeast*, *28*(11), 795-798. https://doi.org/10.1002/yea.1905