

Human Papillomavirus 16 L1 Protein Expression and Self-Assembly in Recombinant *Pichia pastoris*

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Abstract

Human papillomavirus virus-like particles (HPV VLPs) have been shown to be effective in preventing cervical cancer when used as a prophylactic vaccine. However, little attention has been paid to the process of L1 expression and self-assembly of VLPs in vivo. In this study, the methanol concentration is optimized during fermentation to optimize L1 expression and self-assembly into VLPs. HPV 16 L1 was expressed in *Pichia pastoris* GS115, and successfully self-assembled into HPV 16 VLPs. The results demonstrate that there is a strong correlation between methanol concentration and the expression of HPV L1 as well as VLP formation. During the methanol induction phase, low concentrations of residual methanol (0.1-0.3%) promoted the expression of free L1 and increased the total content of L1, whereas a higher residual methanol concentration (0.32-0.6%) was conducive for the self-assembly of L1 into VLPs. To conclude, if the residual methanol concentration is too low or too high, the levels of L1 can easily decrease during the fermentation process.

Keywords: Recombinant *Pichia Pastoris*; Human Papillomavirus Virus-like particles; Methanol concentration optimization; Monomer type 16; Self-assembly

Introduction

Cervical cancer has become the second largest cause of cancer death among women after breast cancer [1]. Human papillomavirus (HPV) is the main etiological agent of cervical cancer, as persistent infection with oncogenic HPV is necessary for the development of cervical cancer. In recent years, several large clinical trials have shown that HPV virus-like particles (VLPs) can be prepared into prophylactic vaccines and provide a sustained immune response against HPV infection. It is estimated that the development of this safe and efficient vaccine can reduce the worldwide incidence of cervical cancer by 70% through the universal immunization of preadolescent girls [2].

The HPV capsid protein consists of the major capsid protein L1 (comprises 80% of the capsid protein) and minor capsid protein L2 [3,4]. L1 monomers can be expressed in eukaryotic systems such as insect and yeast cells and can self-assemble into virus-like particles [5,6]. Due to their strong hydrophobicity, L1 monomers rely on interactions between an α -helix, β -fold, and a β -loop forming a hydrophobic region between the monomers, which combine to form pentamers. Seventy-two pentamers then combine by disulfide bonds to form a complete, highly symmetrical icosahedron, referred to as an HPV virus-like particle (HPV VLP) [7,8]. The assembly of VLPs consisting of HPV L1 is a reversible process. For example, changes in pH, ionic strength, and/or redox potential can result in the depolymerization and reassembly of VLPs [9]. These self-assembling VLPs can mimic the natural virus to a certain extent, which is a critical step in the vaccine development process. The morphology and immunogenicity of the self-assembling VLPs is similar to HPV virions; however, the VLPs lack viral DNA, and therefore do not stimulate potentially harmful oncogenes so as to provide a safe immune response. After injection of the vaccine, the systematic generation of high levels of anti-HPV L1 IgG antibodies can be achieved, providing HPV subtype-specific immunity as well as immunity against related subtypes.

Numerous studies on HPV vaccines have been carried out recent years, with the L1 protein expressed in several expression systems

including *Escherichia coli*, *Saccharomyces cerevisiae*, insect cell-baculovirus system, plant, and *Pichia pastoris* [10-14]. However, due to low yields and relatively high cost, the application of the vaccine in developing countries with high HPV infection rates is limited [15]. It is therefore highly desirable to reduce costs and improve vaccine production. Methanol nutritional type *Pichia* is used in this study because of the powerful genetic techniques available, high expression levels, rapid growth rate on relatively simple media and well-established fermentation technology, coupled with its economy of use [16]. *P. pastoris* can use methanol as its sole carbon source, and can also use methanol to induce the expression of foreign proteins. Therefore, the methanol concentration is one of the key factors that affect protein expression.

Until now, optimized conditions for the expression of HPV VLPs in the yeast *P. pastoris* system have rarely been reported. However, the methanol concentration, pH, and initial inoculum size were shown to affect cell growth and the expression of HPV L6 L1 in *Hansenula polymorpha* culture [17]. Carbon source components and harvest time were found to be key factors which influence the production of HPV 16 L1 in *S. cerevisiae* culture [18]. Moreover, the codon optimized MO-HPV58 L1 gene enhanced the expression of soluble L1 protein in *S. cerevisiae* [19]. Studies on the self-assembly process of L1 were

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conducted *in vitro* [20, 21], and there are currently no reports of *in vivo* studies of the L1 self-assembly process. In this study, we investigated the effects of the methanol concentration in the fermentation process on the expression of HPV 16 L1 and L1 self-assembly into HPV 16 VLPs in *P. pastoris* cells *in vivo*.

Materials and Methods

Strain

Recombinant *Pichia pastoris* GS115/HPV16L1 with the mut^s phenotype was genetically constructed based on the *Pichia* Expression Kit for the expression of recombinant proteins in *Pichia pastoris* (Invitrogen, CA, USA). Briefly, the synthetic 16L1 gene was inserted into pPIC3.5K at the *EcoRI* and *NotI* sites to generate pPIC3.5K-HPV16L1. It was then linearized using *BglII* and electroporated into competent GS115 cells followed by patching onto MD/MM plates. MD medium consisted of 13.4% Yeast Nitrogen Base without Amino Acids (YNB), 4×10⁻⁵% biotin and 2% glucose, and MM consisted of 13.4% YNB, 4×10⁻⁵% biotin and 0.5% methanol. Colonies that grew normally on the MD plate but slowly on the MM plate were of the GS115/pPIC3.5K-HPV16L1 strain with the required His⁺Mut^s phenotype.

Media

YPD media was used as plate and seed media, while the fermentation medium was FM21 basal salt containing glycerol 4% (w/v) and 4 mL/L PTM1 trace element solution. Feed growth medium was 50% glycerol (w/v, containing 12 mL/L PTM1). The fermentation induction medium was 100% methanol containing 12 mL/L PTM1.

Methods

Shake flask culture: In order to maintain consistent physiological characteristics of cells in different shaken flasks, the fermentation broth was taken when the OD₆₀₀ of cells in a 30 L bioreactor reached approximately 200, then 50 mL of broth was inoculated into a 500 mL shake flask and incubated for 2 h to deplete the glycerol. Next, the protein expression phase was initiated by the addition of fermentation induction medium at 12 h intervals.

Fermentation: A single colony was picked from a fresh YPD plate and inoculated into the seed medium, and cultured at 30°C with a shaking speed of 220 rpm for 20 to 25 h until an OD₆₀₀ between 10 and 15 was achieved. The entire 1.5 L/4 L was aseptically transferred into a 30 L/100 L bioreactor containing 15 L/40 L of the fermentation medium. The pH was adjusted to approximately 5.0 with ammonia and kept at 5.0 during the cell growth phase. When the glycerol was completely consumed, dissolved oxygen (DO) levels suddenly increased, followed by a glycerol-fed batch phase. In this phase 50% (v/v) glycerol was fed until an OD₆₀₀ between 350 and 400 was reached. The induction was performed with a continuous feed containing 100% methanol. The feeding rate for methanol was controlled according to the concentration of residual methanol in the fermentation bioreactor. DO levels in the bioreactor were maintained at 20% to 40% for the entire fermentation by 1.0-2.0 VVM aeration and 500 rpm agitation speed. Pure O₂ could be injected if needed. The fermentation period was approximately 72 h to 96 h.

Cell optical density (OD₆₀₀) analysis: The OD₆₀₀ was measured after dilution, and deionized water was used as the control for the colorimetric determination (OD₆₀₀=OD reading×dilution).

Glycerol concentration: The glycerol concentration was analyzed using the sodium periodate titration method as previously described [22].

Methanol concentration: The methanol concentration was detected quantitatively by gas chromatography (GC) analysis as described in our previous publication [23]. FC-2002 was used as an auxiliary to detect the flow of methanol (East China University of Science & Technology, Shanghai, China).

Semi-quantitative analysis of HPV 16 L1: Cells were mechanically lysed using glass beads and the lysate supernatant was analyzed by SDS-PAGE [24], while purified HPV16 L1 with standard concentrations and lysate supernatant samples prior to induction were used as controls. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. A HPV16 L1 monoclonal antibody (purified mouse anti-L1 protein of human papilloma virus; BD Biosciences, USA) was the primary antibody used to recognize and capture HPV16 L1, and this was specific against HPV16 L1. The secondary antibody was an alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Millipore, USA). The relative concentration of monomeric L1 was detected and calculated using a Bio-RAD Chemi Doc XRS with Image Lab Software (Bio-Rad, USA) after using the ECL chromogenic reagent (RPN2232, GE).

Quantitative determination of HPV 16 VLPs (ELISA): Cells were mechanically lysed using glass beads and centrifuged, the lysate supernatant was retained as the samples for analysis. Samples were then diluted with 1% BSA, and 100 μL of each sample was added to the microporous plate and coated with a monoclonal antibody that was specific against VLPs (SHS004, SIBP, China). Lysate supernatant samples before induction were used as the negative control. They were then incubated for 60 min in plates at 37°C. The liquid was then discarded, and the wells were washed with 1× washing liquid three times, before being patted dry.

After washing, 100 μL of enzyme binding material working fluid was added to each well, incubated for 45 min in a membrane-sealed plate at 37°C. Then all liquid was removed and wells were washed with 1× washing liquid five times, and patted dry after the completion of the last wash. After the washing steps, 50 μL of chromogenic substrate solution A and 50 μL of substrate color liquid B was added to each well, mixed gently and incubated at room temperature in the dark for 5 to 10 min. After this, 50 μL of terminate liquid was added to each well and gently mixed. The microplate wavelength was 450 nm, the reference wavelength was 630 nm, and the absorbance value (OD) of each well was measured. The results were fitted with a logX-logY function.

Results

Expression of HPV 16 L1 in methanol-induced recombinant *P. pastoris*

L1 (55 kDa) was expressed and self-assembled into VLPs during the methanol induction phase. Total L1 content was calculated as a relative content and obtained by semi-quantitative Western blotting (Figure 1). Purified HPV 16L1 was used as the standard concentration as a concentration control. Generally, methanol concentration plays a vital role in the expression of foreign proteins in the methylotrophic yeast *P. pastoris* although it is toxic to cells. As shown in Figure 2, in the early methanol induction period, the amount of total L1 rapidly increased when the residual methanol concentration was less than 0.32%. When the residual methanol concentration increased beyond 0.32%, however, the total L1 concentration dropped sharply. Once the residual methanol concentration was <0.3%, the production of total L1 increased again during this production phase. This result suggests that the content of L1 changed according to variations in the residual

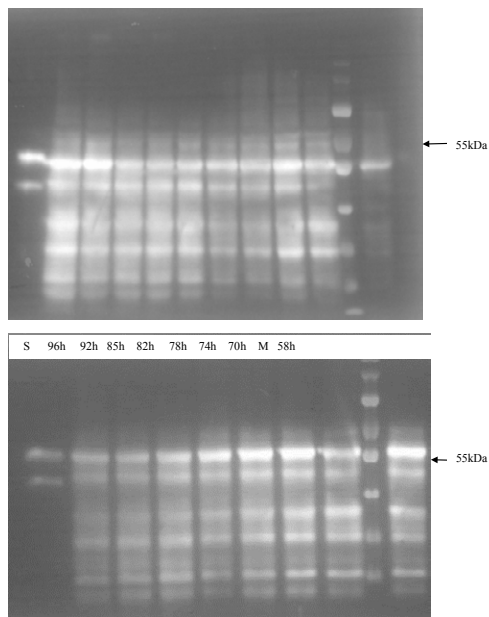


Figure 1: Figure of the Western blot assay to present the expression of HPV 16 L1 in recombinant *Pichiapastoris* GS115/ HPV16L1 cultured in a 100-L bioreactor. Samples from 34 h to 96 h were all lysate supernatants, HPV16 L1 appeared at 55 kDa. 34 h: lysate supernatant sample before induction; M: marker (PageRuler® Prestained Protein Ladder, 10 to 170 kDa, 26616, Thermo Scientific Pierce); S: purified HPV16 L1 with standard concentrations.

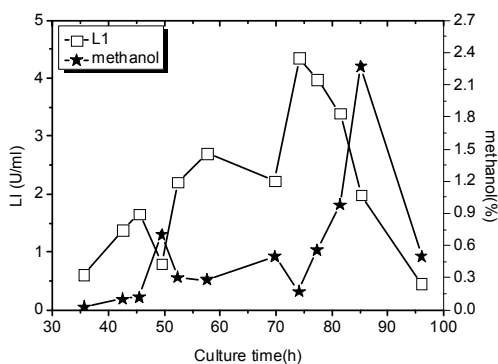


Figure 2: Expression of HPV 16 L1 and methanol concentration in recombinant *Pichiapastoris* GS115/ HPV16L1 cultured in a 100-L bioreactor.

Recombinant *Pichiapastoris* GS115/ HPV16L1 was cultivated in a fed-batch culture. It has two phases: growth phase including a glycerol-batch phase and a glycerol-fed batch phase, and a protein expression phase, which was named the methanol induction phase that was fed with methanol from 35 h. The feed rate was adjusted based on the residual methanol concentration in the broth. "□": HPV16L1 production concentration; "★": residual methanol concentration"

methanol concentration. This demonstrates that the L1 content was closely related to the residual methanol concentration. The threshold value of the residual methanol concentration was approximately 0.32% to achieve a high-level content of L1.

HPV 16 L1 expression and self-assembly during methanol-induced recombinant *P. pastoris* fermentation

During the expression time course of HPV 16 L1 in recombinant *P. pastoris* cultured in a 100-L bioreactor (Figure 3), L1 was expressed and

self-assembled into VLPs with increasing methanol concentrations. When the OD_{600} was less than 360, and residual methanol concentrations were below 0.32%, the content of total L1 and the assembly of VLPs both increased. If the residual methanol concentration was higher than 0.32% (50 h, 0.7%), the L1% (percentage of L1 of the total protein) drastically reduced. Additionally, cell growth was inhibited as observed by a reduction of biomass yield and the concentration of VLPs decreased for depolymerization. When the OD_{600} was greater than 360, the content of total L1 increased rapidly, provided the residual methanol concentration was maintained below 0.3%. Furthermore, the VLPs depolymerized, which means that the level of free L1 expression increased. Possible reasons for this finding are that low concentrations of methanol might only be sufficient for cell growth and L1 expression, and not for VLP self-assembly. If the residual methanol concentration stayed higher than 0.3%, the total L1 content decreased, VLP assembly increased and the L1% dropped, which indicated that the level of free L1 expression decreased.

It was interesting to note that the number of VLPs would not increase until total L1 were reduced to below 2 U/mL. This might reflect that a minimum concentration of free L1 is required for self-assembly. Residual methanol concentration strictly controlled the expression of L1, and also affected the relationship between L1 and VLPs. Therefore, it was necessary to optimize the residual methanol concentration during *P. pastoris* fermentation to express HPV L1.

Optimization of methanol concentrations for expression of HPV 16 VLPs during recombinant *P. pastoris* fermentation

Initial methanol concentrations of 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.8%, and 1.0% (v/v) were tested for the expression of HPV 16 L1 in flask culture. As shown in Figure 4, the content of L1 reached a maximum at a methanol concentration of 0.2%, while optimal cell growth conditions we observed at 172 (OD_{600}) with a methanol concentration of 0.3%. Once the initial methanol concentration was higher than 0.4%, the L1 content decreased rapidly due to the degradation (L1%) or formation of insoluble aggregates. However,

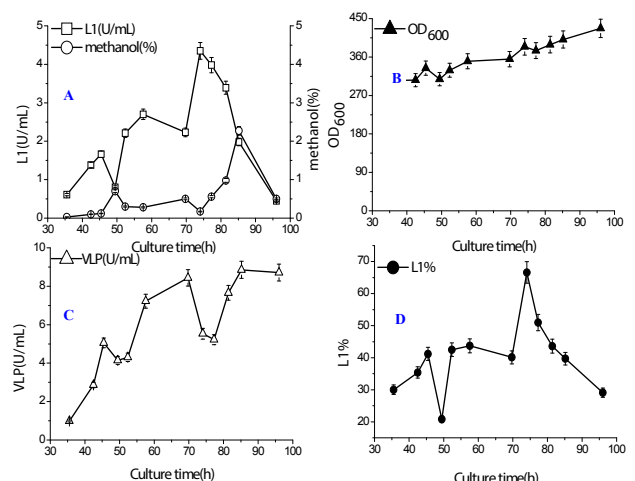


Figure 3: Time-course of HPV 16 L1 expression by recombinant *Pichiapastoris* GS115/ HPV16L1 fermentation in a 100-L bioreactor. A: HPV 16 L1 production during methanol induction phase (□: HPV16L1 production concentration; ○: residual methanol concentration); B: cell growth during the methanol induction phase (▲: cell optical density determined at the wavelength of 600 nm); C: virus-like particle (VLP) level during the methanol induction phase (Δ: VLP concentration); D: HPV 16 L1 percentage during the methanol induction phase (●: HPV 16 L1 percentage).

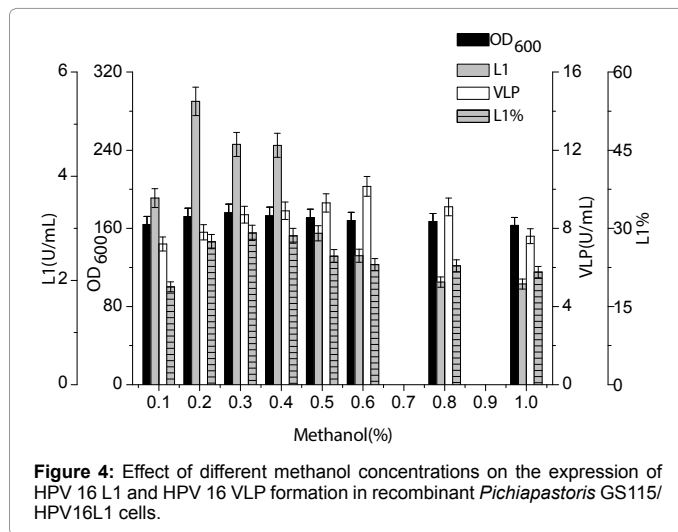


Figure 4: Effect of different methanol concentrations on the expression of HPV 16 L1 and HPV 16 VLP formation in recombinant *Pichiapastoris* GS115/HPV16L1 cells.

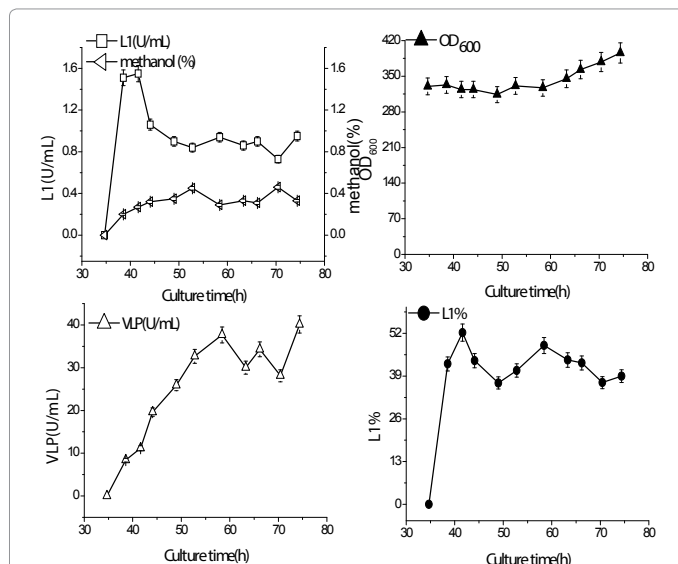


Figure 5: Expression of HPV 16 L1 by recombinant *Pichiapastoris* GS115/HPV 16 L1 cultured in a 30-L bioreactor during the methanol induction phase. A: HPV 16 L1 production during the methanol induction phase (□: HPV 16 L1 production concentration; ◁: residual methanol concentration); B: cell growth during the methanol induction phase (▲: cell optical density determined at the wavelength of 600 nm); C: virus-like particle (VLP) level during the methanol induction phase (Δ: VLP concentration); D: HPV 16 L1 percentage during the methanol induction phase, (●: HPV16L1 percentage).

VLP assembly showed different optimal conditions, as higher residual methanol concentrations were more conducive to VLP assembly. The initial concentration of 0.6% appeared to be optimal, while the residual methanol was measured at 0.35% (Table 1). When the initial methanol concentration was higher than 0.8% while the residual methanol concentration was 0.61%, the content of VLPs decreased. This finding strongly suggests that the residual methanol concentration is closely related to the expression of HPV L1 and the assembly of HPV VLPs. In summary, residual methanol concentrations that range from 0.1% to 0.3% optimally increase the total L1 content, while concentrations of 0.35% to 0.6% improve VLP self-assembly.

Expression of HPV 16 L1 in recombinant *P. pastoris* cultured in a 30 L bioreactor

To verify the influence of residual methanol concentrations on L1

Initial methanol (%)	Residual methanol (%)	Content of total L1(U/ml)	Concentration of VLPs(U/ml)	L1%
0.1	0	3.61	7.22	17.06
0.2	0	5.35	7.81	27.38
0.3	0.08	4.59	8.73	29.87
0.4	0.19	4.53	8.91	28.96
0.5	0.3	2.92	9.29	24.31
0.6	0.35	2.37	10.15	22.85
0.8	0.61	1.96	9.12	22.71
1.0	0.86	1.91	7.63	20.52

Table 1: Influence of methanol concentration on recombinant *Pichia pastoris* expression of HPV 16 L1.

expression and self-assembly into VLPs, fermentation was carried out in a 30 L bioreactor. The strategy was to maintain residual methanol concentrations below 0.3% in the early induction period to enhance the content of L1, while later increasing the residual methanol concentration to between 0.3% and 0.5% to promote L1 self-assembly into VLPs. Similarly, residual methanol concentration affected L1 expression and self-assembly (Figure 5A and 5B). When the residual methanol concentration was kept below 0.3%, L1 was expressed quickly and the total L1 reached a maximum while simultaneously self-assembling into VLPs. When the residual methanol concentration was kept between 0.32% and 0.45%, L1 content decreased sharply and the L1% dropped rapidly. Furthermore, the content of VLPs rose, which confirmed that the expression of free L1 decreased. As the observed rate of VLP concentration showed a distinct linear upward trend, the L1 assembly speed was so rapid that some insoluble aggregates might have formed due to incomplete or improper folding, so as to reduce the total concentration of L1. After that, cells began to grow rapidly and the VLP content remained stable. It is possible that, at this point, there was no further expression of free L1.

This experiment provides strong evidence that lower concentrations of residual methanol (0.1–0.3%) promoted L1 expression and a higher concentration of residual methanol (0.32–0.6%) effectively promoted L1 monomer self-assembly into VLPs. The L1 content can be sensitive to the residual methanol concentration. As such, the feed strategy according to the optimized residual methanol concentrations can be applied to improve the expression of L1 and VLP production.

Discussion

In this study, we investigated the effects of residual methanol concentration on the expression of HPV L1 during fermentation and self-assembly of L1 monomers into VLPs using recombinant *P. pastoris*. When *P. pastoris* is used for the expression of exogenous protein, methanol acts both as a carbon and energy source for cell growth and metabolism, as well as an induction signal for recombinant gene expression. Suboptimal methanol concentrations in the fermentation broth are not conducive to the expression of the exogenous protein. Effective control of the residual methanol concentration in the process of induction culture will significantly affect the efficient expression of HPV L1 in the *P. pastoris* expression system.

We induced cultures in both 30 L and 100 L bioreactors, and discovered a strong correlation between L1 expression, VLP self-assembly and residual methanol concentration (Figure 1, Figure 4A), and that L1 expression was sensitive to changes in residual methanol concentration. Low residual methanol concentrations can promote the expression of L1. Once the residual methanol concentration was greater than 0.32%, the L1 content dropped sharply. Some studies suggest that proteases and lack of a nitrogen source are the primary

causes of recombinant protein degradation [25,26]. On the other hand, L1 monomers formed some larger insoluble polymers and aggregates during the self-assembly process, which were removed by centrifugation after cell disruption [18], and resulted in a decrease in total L1 content. When residual methanol concentration was maintained above 0.32%, although the L1 content was reduced, the yield of HPV VLPs increased. At high residual methanol concentrations, L1 monomers were more conducive to the assembly of VLPs. Possible reasons were that higher residual methanol concentrations in the culture could meet the energy demand for α -helix, β -folding and disulfide-bond formation in the L1 self-assembly process. The optimization of methanol concentration experiments in shake flasks and the verification in 30 L bioreactor culture further confirmed that low residual methanol concentrations are conducive to L1 expression and a higher concentration of residual methanol will promote L1 self-assembly into VLPs (Figure 3). Also, the feed strategy along with optimized methanol concentrations can effectively improve the production of L1 and VLPs.

When the initial methanol concentration was too low (<0.2%), methanol was quickly consumed and became the rate-limiting substrate, so that the carbon source and energy requirements for cell growth and L1 expression could not be fulfilled. The methanol was used by *P. pastoris* primarily for cell growth and not for the expression of L1. Simultaneously, L1 degraded more rapidly and the total content was lower. When too much methanol was added, residual concentrations of methanol increased accordingly. However, high residual methanol concentrations (>0.32%) could inhibit the activity of some key enzymes in the process of methanol metabolism, such as alcohol oxidase, which not only restrained cell growth but also reduced the expression of L1 [27].

Intracellular free L1 would no longer self-assemble into VLPs below a threshold concentration during the fermentation process. Ding et al. simulated the assembly and aggregation of VLPs in vitro [28], and found that there was a critical concentration required for the assembly (also called the minimum assembly concentration) of capsomeres. When the capsomere concentration dropped below a certain value, VLP assembly ceased. Mukherjee et al. (HPV 16 VLPs), and Casini et al. [29] (HPV 11 VLPs) also mentioned this critical assembly concentration in their studies on VLP assembly in vitro. Therefore, improving the concentration of free L1 is an encouraging strategy to increase the yield of HPV VLPs.

Conclusion

In this study, we have determined an optimal fermentation strategy for production of HPV VLPs. During the early induction period, lower residual methanol concentrations (0.1-0.3%) are used to rapidly express and accumulate L1, and increase the cell biomass. Then, increasing residual methanol concentrations to between 0.32% and 0.6% promotes L1 self-assembly into HPV VLPs. This research lays an important foundation for fermentation optimization for the production of HPV VLPs and provides a greater understanding of the expression of intracellular L1 as well as the characteristics of L1 self-assembly into VLPs. Improving the yield of HPV VLPs is a significant step toward reducing the cost of the HPV vaccine, and will benefit developing countries with high HPV infection rates.

Authors' Contributions

Jing Zhao carried out the fermentation and data statistic; Zejian Wang participated in its experimental design, Haifeng Hang participated in on-line fermentation data processing and analysis,

Meijin Guo participated in its design and drafted the manuscript, Yingping Zhuang participated in its design and coordination, Ju Chu critically revised the manuscript, Siliang Zhang participated in analysis and interpretation of data, and Jueren Lou carried out strain construction and final manuscript. All authors read and approved the final manuscript.

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