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# Medium Optimization for Recombinant Human Papillomavirus Type 52 L1 Protein Production in *Pichia pastoris* GS115 Platform on Bioreactor Scale

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## ABSTRACT

Human papillomavirus (HPV) stands as the primary etiological agent in the development of invasive cervical cancer worldwide. The L1 protein is a pivotal constituent of prophylactic HPV vaccines. Notably, HPV type 52 is one of the most prevalent genotypes found in squamous cell carcinoma cases in Indonesia. This research endeavor aims to enhance the productivity of recombinant HPV-52 L1 protein by optimizing the culture conditions of *P. pastoris* GS115 cells. In this study, we conducted trials employing 17 different media variants to optimize the expression of recombinant HPV-52 L1 protein. The results from small-scale experiments revealed three media, namely SYN6.10, BMMY, and SYN6.1, which exhibited promising yields of recombinant HPV-52 L1 protein as assessed through ELISA or immunoassay analysis. We succeeded in refining the SYN6.10 derivative, denoted as SYN6.10b, specifically designed for use in 1-L and 5-L bioreactors. This achievement was realized by adjusting Trace Element Solution (TES) and Vitamin Solution (VS) concentrations and implementing a methanol fed-batch phase with the addition of 0.3% methanol after 24 and 48 hours of fermentation in the *P. pastoris* medium. Further visualizations through SDS-PAGE and western blot analysis confirmed the protein after 72 hours of fermentation in a 1-L bioreactor using the SYN6.10b medium. In conclusion, the SYN6.10b medium required a 72 hours fermentation period to successfully express recombinant HPV-52 L1 protein in the *P. pastoris* platform.

## 1. Introduction

Cervical cancer ranks second among cancers in women living in poor and developing countries, with an estimated 569,847 cases in a year (Bray *et al.* 2018; WHO 2020). More than 200 HPV genotypes have been identified, with HPV 16, 18, 31, 33, 45, 52, and 58 being high-risk genotypes as the cause of cervical cancer (Clifford *et al.* 2003; Al Adawiah *et al.* 2024). HPV genotype 52 is known to be the most common type

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found in Indonesia and some countries in East Asia (Vet *et al.* 2008).

Currently, three HPV vaccines have obtained licenses, and all are based on virus-like particles (VLP) from HPV L1 capsid protein. Two of them, Gardasil® (based on L1 protein from HPV types 6, 11, 16, and 18) and Gardasil 9® (based on L1 protein from HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58) are produced by yeast (*Saccharomyces cerevisiae*). In contrast, the other one, Cervarix (based on L1 protein from HPV types 16 and 18), is produced by a baculovirus expression system (Eto *et al.* 2021). Nonetheless, the high production costs result in a steep selling price for all three, rendering them largely unaffordable for the majority of women in developing nations. Hence, there is an urgency to develop more cost-effective alternative vaccines to expedite and broaden vaccination coverage.

Dewi *et al.* (2023) found that the expression of recombinant HPV-52 L1 proteins can be effectively achieved using yeast as a host. Among yeast hosts, *P. pastoris*, a methylotrophic yeast, is a prospective expression system as a host in producing recombinant proteins due to its prominent properties compared to other eukaryotic or prokaryotic hosts, i.e. rapid cell growth followed with high cell density, cost-effective growth, making it favorable for large-scale production, genetic manipulation feasibility, capacity for multi post-translational modifications and lower glycosylation levels compared to other yeasts, which affects protein stability and immunogenicity (De Schutter *et al.* 2009).

Optimization of HPV 52 L1 protein expression using methylotrophic yeast *P. pastoris* GS115 strain as a host has been previously documented (Dewi *et al.* 2023; Firdaus *et al.* 2023). Enhancing the production of recombinant protein is the major focus in the manufacturing of vaccine materials. One effective approach to boost protein production yield is through media optimization, as the media directly influences growth rates and cell viability. The commonly used media for cultivating *P. pastoris* is Basal Salt Medium (BSM), which consists of biotin, ammonium hydroxide, methanol (MeOH), or glycerol as carbon sources and trace elements. In this medium, *P. pastoris* is required to synthesize all metabolic intermediates, resulting in slower growth compared to the use of richer, complex media like BMGY (Buffered Glycerol- complex Medium) and BMMY (Buffered MeOH-complex Medium). In addition, this medium has a disadvantage because it causes salt precipitation (Safder *et al.* 2018). Matthews *et al.* (2018) have optimized culture media by using a richly complex medium (Generation 1 medium (25 mM ammonium, 5 mM glutamine, 5 mM arginine, and vitamin solution)) to accelerate growth rate and protein production in *P. pastoris* to increase protein expression in *P. pastoris*. However, while rich, complex media effectively elevate cell density and productivity, their usage can significantly inflate production costs, especially when scaled up for large-scale production.

The scale-up of HPV L1 protein production in bioreactors has been explored in our previous studies (data not shown). These investigations have illuminated the process of expressing the HPV type 52 L1 protein in bioreactors, employing a VLP expression system based on Hansenula polymorpha. In addition, Bredell *et al.* (2018) successfully enhanced the production of chimeric L1/L2 HPV 16 protein by *P. pastoris* on a bioreactor scale using a rich complex medium (BMGY). However, the optimization of L1 HPV 52 protein production remains to be elucidated. The current study focused on the scale-up production of L1 HPV 52 protein expression in *P. pastoris* using a bioreactor system with a notable shift from a rich complex medium to a minimal medium. This study endeavors to enhance protein production by achieving substantial biomass using the SYN6.10 medium while simultaneously improving the cost-effectiveness of the bioreactor system.

## 2. Materials and Methods

### 2.1. Yeast Strain, Media, and Reagents

The methylotrophic yeast *P. pastoris* GS115 strain PD902 L1 HPV 52 truncated colony 6 was used in this study. The gene encoding the L1 Protein was synthesized by ATUM Company DNA 2.0 Gene Design & Synthesis (California, USA), inserted into pD902, and transformed to *P. pastoris* using the Groningen method (Dewi *et al.* 2023; Rosmeita *et al.* 2023). YPD (Yeast Extract Peptone Dextrose) medium was used as the cultivation medium with composition (g/L): 1% yeast extract, 2% peptone, 2% dextrose, and the pre-expression medium was BMGY (1% yeast extract, 2% peptone, 100mM potassium phosphate pH 6.0, 1.34% YNB, 1% glycerol, 0.2% Biotin). The variation of media composition (BMMY, SYN6, and BSM), enrichment, and inducer used for recombinant protein expression are shown in Table 1.

### 2.2. Cultivation in Flask Scale

The recombinant *P. pastoris* cells were grown in the test tube containing 5 ml of YPD and 100 µg/ml

Table 1. Medium optimization in the fed-batch fermentation

| Medium | Composition | Biomassa (g) | $OD_{600}$ | ELISA (µg/µL) | References |
|---|---|---|---|---|---|
| SYN6.1 | $(NH_4)_2SO_4$ 7.66 g, KCl 3.3 g, $KH_2PO_4$ 1 g, $MgSO_4$•$7H_2O$ 3 g, NaCl 0.3 g, *Me 1%, *VS 1%, *TES 1%, 4% (v/v) glycerol, peptone (2%) 1%, $CaCl_2$ 1%, MeOH 1% | 0.51 | 9.48 | 0.320 | This study |
| SYN6.2 | $(NH_4)_2SO_4$ 7.66 g, KCl 3.3 g, $KH_2PO_4$ 1 g, $MgSO_4$•$7H_2O$ 3 g, NaCl 0.3 g, *Me 1%, *VS 1%, *TES 1%, glycerol (50%) 4%, peptone (2%) 1%, $CaCl_2$ 1%, MeOH 10% | 0.53 | 6.98 | 0.280 | This study |
| SYN6.3 | $(NH_4)_2SO_4$ 7.66 g, KCl 3.3 g, $KH_2PO_4$ 1 g, $MgSO_4$•$7H_2O$ 3 g, NaCl 0.3 g, *Me 1%, *VS 1%, *TES 1%, glycerol (50%) 4%, peptone (2%) 1%, $CaCl_2$ 1%, 10 ml/L [MeOH 10%, glycerol 10% dan peptone 20%] | 1.74 | 23.52 | 0.306 | This study |
| BSM1.4 | $H_3PO_4$: 26.7 ml; $K_2SO_4$: 18.2 g; $CaSO_4$: 0.93 g; $(MgSO_4)$•$7H_2O$: 14.9 g; KOH: 4.13 g; $C_3H_8O_3$: 40 g; 50% Glycerol: 4%; MeOH: 1%; PTM1 12 ml/L | 0.86 | 32.72 | 0.117 | Bredell *et al.* 2018; Farinha *et al.* 2022 |
| BSM2.5 | $(NaPO_3)_6$: 25 g; $K_2SO_4$: 18.2 g; $CaSO_4$: 0.93 g; $(MgSO_4)$•$7H_2O$: 14 g; $(NH_4)_2SO_4$: 9 g; $C_3H_8O_3$: 40 g; 50% Glycerol: 4%; MeOH: 1%; PTM1 12 ml/L | 0.77 | 19.98 | 0.112 | Gellermann *et al.* 2019 |
| SYN6.6 | $(NH_4)_2SO_4$: 7.66 g; KCl: 3.3 g; $KH_2PO_4$: 1 g; $MgSO_4$•$7H_2O$: 3 g NaCl: 0.3 g; Microelemet: 0.5 %; $CaCl_2$: 2%; 20 ml/L (20% pepton, 20% YE, 10% glicerol, 10%) | 1.41 | 9.28 | 0.307 | This study |
| SYN6.7 | $(NH_4)_2SO_4$: 7.66 g; KCl: 3.3 g; $KH_2PO_4$: 0.5 g; $MgSO_4$•$7H_2O$: 1.5g; NaCl: 0.3 g; Microelemet: 1%; VS: 1%; TES: 1%; 50%; Glycerol: 4%; 2% Pepton: 1%; $CaCl_2$: 1%; MeOH: 1% | 1.05 | 13.7 | 0.295 | This study |
| SYN6.8 | $(NH_4)_2SO_4$: 3.83 g; KCl: 3.3 g; $KH_2PO_4$: 1.5 g; $MgSO_4$•$7H_2O$: 3 g; NaCl: 0.3 g; Microelemet: 1%; VS: 1%; TES: 1%; 50% Glycerol: 4%; 2% Pepton: 1%; $CaCl_2$: 1%; MeOH: 1% | 0.73 | 9.84 | 0.305 | This study |
| SYN6.9 | $(NH_4)_2SO_4$: 7.66 g; KCl: 3.3 g; $KH_2PO_4$: 1 g; $MgSO_4$.$7H_2O$: 3 g; NaCl: 0.3 g; Microelemet: 1%; 50% Glycerol: 4%; 4% Pepton: 1%; CaCl2: 2% | 1.1 | 14.92 | 0.286 | This study |
| SYN6.10 | $(NH_4)_2SO_4$: 7.66 g; KCl: 1.65 g; $KH_2PO_4$: 1 g; $MgSO_4$•$7H_2O$: 3 g; NaCl: 0.15 g; Microelemet: 1%; VS: 1%; TES: 1%; 50% Glycerol: 4%; 2% Pepton: 1%; $CaCl_2$: 1%; MeOH: 1% | 0.88 | 12.48 | 0.524 | This study |
| SYN6.11 | $(NH_4)_2SO_4$: 7.66 g; KCl: 1.65 g; $KH_2PO_4$: 1 g; $MgSO_4$•$7H_2O$: 3 g; NaCl: 0.15 g; Glukosa: 25 g; Microelemet: 1%; VS: 1%; TES: 1%; $CaCl_2$: 1%; MeOH: 1% | 0.67 | 14.86 | 0.259 | Knol *et al.* 2007 |
| BSM2.12 | $(NaPO_3)_6$: 10 g; $K_2SO_4$: 8.2 g; $CaSO_4$: 0.43 g; $(MgSO_4)$•$7H_2O$:4 g; $(NH_4)_2SO_4$: 13 g; $C_3H_8O_3$: 20 g; 50% Glycerol: 4%; MeOH: 1%; PTM1 12 ml/L | 0.7 | 22 | 0.104 | This study |
| BSM2.13 | $(NaPO_3)_6$: 10 g; $K_2SO_4$: 28.2 g; $CaSO_4$: 0.43 g; $(MgSO_4)$•$7H_2O$: 24 g; $(NH_4)_2SO_4$: 4 g; $C_3H_8O_3$: 20 g; 50% Glycerol: 4%; MeOH: 1%; PTM1 12 ml/L | 0.67 | 20.14 | 0.146 | This study |
| BSM2.14 | $(NaPO_3)_6$: 10 g; $K_2SO_4$: 8.2 g; $CaSO_4$: 1.43 g; $(MgSO_4)$•$7H_2O$:4 g; $(NH_4)_2SO_4$: 4 g; $C_3H_8O_3$: 20 g; 50% Glycerol: 4%; MeOH: 1%; PTM1 12 ml/L | 0.73 | 19.98 | 0.124 | This study |

*TES: Trace Element Solution, VS: Vitamin Solution, Me: Micronutrients

Table 1. Continued

| Medium | Composition | Biomassa (g) | $OD_{600}$ | ELISA (µg/µL) | References |
|---|---|---|---|---|---|
| BSM2.15 | Glukosa: 25 g; $CaCl•2H_2O$: 1 g; $K_2SO_4$: 18 g; KOH: 4 g; $(MgSO_4)•7H_2O$: 14 g; $H_3PO_4$: 26 ml; TES: 8 ml/l; VS: 8 ml/l; $H_2SO_4$ (30%) 5 ml/l; MeOH: 1%; PTM1 12 ml/L | 0.85 | 26.84 | 0.133 | Knabben *et al.* 2010 |
| SYN6.16 | $(NH_4)_2SO_4$: 7.66 g; KCl: 3.3 g; $KH_2PO_4$: 1 g; $MgSO_4•7H_2O$: 3 g; NaCl: 0.15 g; 50% Glycerol: 4%; Microelemet: 0.5%; 2% Pepton: 1%; $CaCl_2$: 1%; 20 ml/L (20% pepton, 20% YE, 10% glicerol, 10% MeOH) | 0.69 | 4.56 | 0.285 | This study |
| BMMY | Yeast extract 1%, peptone 2%, yeast nitrogen base 1.34%, potasium fosfat (pH 6) 10%, biotin: $4 \times 10^{-5}$%, MeOH, $ddH_2O$ | 1.41 | 30.08 | 0.395 | This study |

*TES: Trace Element Solution, VS: Vitamin Solution, Me: Micronutrients

Zeocin and incubated in an incubator shaker (TAITEC BR-43FM) at 30°C 250 rpm until $OD_{600}$ reached ~2-6. Then, 1 ml of the culture was inoculated to 25 ml BMGY and incubated under the same conditions for 24 h with aeration of 1:10 to the total volume. After incubation, the cells were centrifuged (CR21GIII Series-High Speed) at 3,000 x g 4°C for 5 min, resuspended in 17 variations of expression medium 25 ml, and incubated at 30°C with shaking at 250 rpm. To induce expression of the recombinant protein from *P. pastoris*, various inducers (Table 1) were added every 24 hours, followed by culture sampling to analyze the optical density.

The cells were harvested after 72 h of growth, centrifuged at 3,000 x g for 5 min, and lysed according to previously reported methods (Dewi *et al.* 2023), with slight modifications. The harvested cultures were resuspended in lysis buffer (50 mM sodium phosphate, 1 mM EDTA, 5% glycerol, 1 mM PMSF, pH 7.4) mixed with an equal volume of 0.5 mm (Ø) glass beads (Merck) and lysed with vortex for 30 seconds followed by incubation on ice for 30 seconds within eight cycles. Supernatants were pooled through centrifugation at 12,000 rpm for 15 min, 4°C, and stored at -20°C prior to examination.

## 2.3. Mini Bioreactor and 1-L Bioreactor Cultivation

The bioreactor-scale fermentation of recombinant *P. pastoris* was initially conducted in a 150 ml working volume mini bioreactor system (Eppendorf DASGIP®, USA). The bioreactor was equipped with a built-in digital controller for pH, temperature, agitation, and dissolved oxygen (DO). Seed culture was prepared in a 10 ml flask scale and then transferred to BMGY in a 100 with the same fermentation parameters for 16 h to continue the fermentation process at the bioreactor scale using BMMY, SYN6.1, and SYN6.10 medium with 150 ml working volume. Culture conditions were as follows: the temperature was maintained at 30°C, agitation 250 rpm, the DO concentration was kept at 30%, pH 6.0 for BMMY, and SYN6.1 and SYN6.10 medium. Induction was carried out every 24 hours. About 4 mL of culture samples were collected for $OD_{600}$ measurement or biomass quantification. Aliquots of the culture were centrifuged at 12,000 rpm for 10 minutes. The resulting pellets were weighed under wet conditions and then dried in an oven at 60°C for 16 h to determine the biomass. Separately, cells were harvested by centrifuged at 6,000 rpm 4°C for 20 min. Cell lysis was performed using a tissue lyser (TissueLyser II, Qiagen, USA) at 20 oscillations per second for 2 min in two cycles. The resulting supernatants were collected and stored at -80°C for further analysis. Fermentation in a 1-L bioreactor (BioFlo® 120-Eppendorf) was conducted using the SYN6-10 medium modification methods of Mousa *et al.* (2012). The different inducer treatment and fermentation conditions at this step are shown in Table 1.

## 2.4. Batch Fermentation in a 5-L Bioreactor

Batch cultivations were carried out in a 5-L bioreactor (BioFlo® 120-Eppendorf) with a 3-L working volume. The basal salt medium was sterilized in a bioreactor at 121°C for 15 minutes. The pH of the culture medium was adjusted to 5.5 using phosphoric acid ($H_3PO_4$) and ammonia solution ($NH_3$). Sterile-filtered trace elements ($CuSO_4•5H_2O$ 6.0 g/L, NaI 0.08 g/L, $MnSO_4•H_2O$ 3.0 g/L, $Na_2MoO_4•2H_2O$ 0.2 g/L, $H_3BO_3$ 0.02 g/L, $CoCl_2$ 0.5 g/L, $ZnCl_2$ 20.0 g/L, $FeSO•7H_2O$ 65 g/L, Biotin 0.2 g/L, $H_2SO_4$ 5 ml, water to a final volume of 1 L)

were transferred to the medium. The inoculum load was adjusted based on the initial concentration of the expression medium ($OD_{600}$ = 0.5), and then the bioreactor was programmed to run at pH 5.5, 30, 400 rpm, and DO at 30%. The culture was supplemented with 0.3% of MeOH (50%, v/v) during 24 and 48 hours of incubation, followed by cell harvesting at 72 h period.

### 2.5. SDS–PAGE, Dot Blot and Western Blotting Analysis

Characterization of HPV 52 L1 expression levels in *P. pastoris* was analyzed by Sodium Deodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot. 30 µL of lysate samples were mixed with the 15 µL Laemmli sample buffer and boiled for 10 min at 95°C. Proteins were fractionated on 12% SDS-PAGE and stained with Coomassie Blue (Sambrook and Russell 2006). Subsequently, qualitative analysis was performed using the Dot Blot method. Fractionated proteins were transferred to nitrocellulose membranes with Bio-Rad Mini Trans-BlotVR (Bio-Rad, USA) at 400 mA per gel for 100 min in a transfer buffer. The membrane was then blocked using skim milk for 1 h and incubated with 5 × $10^{-4}$ primary antibodies and secondary antibodies for another 1 h. Finally, the membrane was incubated with the Nitro Blue Tetrazolium Chloride/5-Bromo-4-chloro-3-indolyphosphate NBT/BCIP chromogenic substrate (Thermo Fisher Scientific, USA) for color development. After color visualization, the membrane was washed using Tris-buffered saline containing 0.1% Tween 20 (TBST) 20 mM Tris, pH 7.4, 150 mM NaCl).

Western blot was performed following the method of Towbin *et al.* (1979), with modifications. The modifications included the use of a Bio-Rad Mini Trans-Blot® system operated at a constant current of 100 mA per gel for 100 minutes instead of the original constant voltage setup. Briefly, the rabbit anti-HPV-52 L1 antibody (Creative Diagnostics, USA) and alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG antibody (Invitrogen, USA) were used as the primary and secondary antibodies, respectively. Fractionated proteins with SDS-PAGE method were transferred to a nitrocellulose membrane with Bio-Rad Mini Trans-BlotVR at 100 mA per gel for 100 min in transfer buffer (25 mM Tris base, 192 mM Glycine, 20% MeOH). The membrane was blocked with TBST supplemented with 5% skim milk for 1 h, washed with TBST, and incubated with a specific antibody for 2 h at room temperature. After incubation, the membrane was washed with TBST three times at room temperature, and the proteins were visualized using an AP chromogenic substrate NBT/BCIP (Thermo Fisher Scientific, USA).

### 2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

The HPV 52 L1 protein was quantified by ELISA using specific antibodies (Creative Diagnostic, CABT B8799). Briefly, the ELISA plate was coated with the anti-L1 HPV 52 antibody overnight at 4°C. The coated plate was washed three times with washing buffer (PBST; 0.05% Tween-20 in PBS) and blocked with blocking buffer (7.5% Bovine Serum Albumin; Sigma Aldrich in PBST) for 1 h at room temperature. Unabsorbed proteins were removed by wash buffer. After washed three times with 200 uL wash buffer, 0.005% anti-HPV 52 L1 as a primary antibody was added and incubated for another 2 h at room temperature. The unbound primary antibodies were removed by washing. The anti-mouse IgG-HRP conjugate was diluted with PBST 0.05%, Tween-20 in PBS, and 7.5% BSA was added to the well, incubated for 2 h at room temperature, and washed with wash buffer three times. The proteins were stained with the substrate ABTS (Thermo Fisher Scientific, USA) by incubating at room temperature for 45 minutes, and the optical density was detected using a microplate reader (Varioskan, Thermo Fisher Scientific, USA) at 405 nm and 620 nm. An anti-L1 HPV 52 antibody (Creative Diagnostics, USA) was used as a positive control.

### 2.7. Statistics

All experiments were performed in triplicate, and data were statistically analyzed using a t-student test followed by ANOVA. All values are expressed as mean ± standard deviation.

## 3. Results

### 3.1. Expressions of L1 HPV 52 Protein in the Different Media

The selection of the medium for expressing the L1 HPV 52 protein began with a Box-Behnken design featuring 6 factors and 2 responses, namely biomass and optical density, which involved 46 experiments (data not shown). Design Expert 13.0.5.0 software from Stat-Ease Corporation, USA, was employed for the analysis and prediction of the optimal conditions, leading to the development of 17 different media variations (Table 1). Consequently, these media were

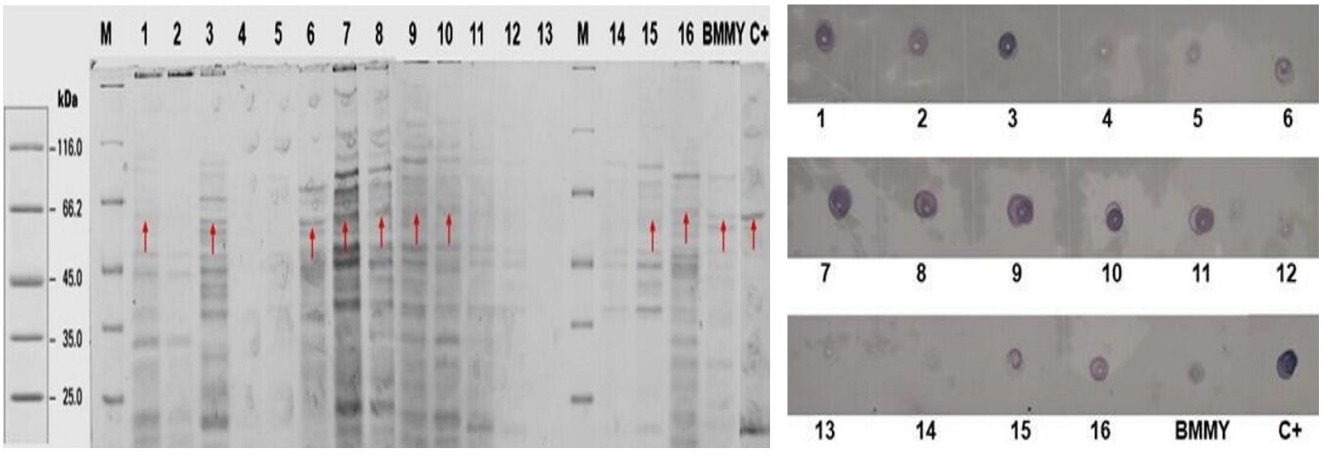
utilized for flask-scale cultivation in the subsequent experiments. Proteins were qualitatively analyzed using SDS-PAGE and Dot-blot, which revealed that the L1 HPV 52 protein (~55 kDa) was well expressed in media No. 1, 3, 6, 7, 8, 9, 10, 11, 15, and BMMY (Figure 1). Among the ten media that show a good expression of the L1 HPV 52 protein, SYN6 media were predominant.

### 3.2. VLP L1 HPV 52 Mini Bioreactor Expression

Optimization results from 17 flask-scale (batch) experiments yielded three media with high cell density ($OD_{600}$) and protein yield through ELISA analysis, namely SYN6.1, SYN6.10, and BMMY. Further testing was conducted using these three media at the mini bioreactor scale. Table 2 shows that SYN6.10 media at the mini bioreactor scale resulted in higher cell density or $OD_{600}$ (7.83) and ELISA (0.0067 µg/50 µL) values compared to SYN6.1 and BMMY after 48 hours of fermentation. SYN6.10 was recommended as the production medium.

### 3.3. VLP L1 HPV 52 1-L Bioreactor Production

The results obtained from previous batches were used as an evaluation material to improve cell densities ($OD_{600}$) and biomass, serving as indicators for increased production of recombinant HPV 52 L1 protein. Bioreactor-scale fermentation of recombinant *P. pastoris* was initially conducted using a 1-L bioreactor (Eppendorf BioFlo® 120).




A B

Figure 1. SDS-PAGE 55 kDa shown red line (A), and dot-blot (B) analysis of HPV 52 L1 expression *P. pastoris* on different media (1 = SYN6.1; 2 = SYN6.2; 3 = SYN6.3; 4 = BSM1.4; 5 = BSM1.5; 6 = SYN6.6; 7 = SYN6.7; 8 = SYN6.8; 9 = SYN6.9; 10 = SYN6.10; 11 = SYN6.11; 12 = BSM2.12; 13 = BSM2.13; 14 = BSM2.14; 15 = BSM2.15; 16 = SYN6.16)

Table 2. Composition of three media used in mini bioreactor based on flask scale optimization

| Medium | Composition | Biomassa (g) | $OD_{600}$ | ELISA (µg/µL) |
|---|---|---|---|---|
| SYN6.1 | $(NH_4)_2SO_4$ 7.66 g, KCl 3.3 g, $KH_2PO_4$ 1 g, $MgSO_4 \cdot 7H_2O$ 3 g, NaCl 0.3 g, *Me 1%, *VS 1%, *TES 1%, 4% (v/v) glycerol 50%, peptone (2%) 1%, $CaCl_2$ 1%, MeOH 1% | 0.0027 | 5.00 | 0.0030 |
| SYN6.10 | $(NH_4)_2SO_4$ 7.66 g, KCl 1.65 g, $KH_2PO_4$ 1 g, $MgSO_4 \cdot 7H_2O$ 3 g, NaCl 0.15 g, *Me 1%, VS 1%, TES 1%, 4% (v/v) glycerol 50%, peptone (2%) 1%; $CaCl_2$ 1%, MeOH 1% | 0.0029 | 7.83 | 0.0067 |
| BMMY | Yeast extract 1%, peptone 2%, yeast nitrogen base 1.34%, potassium fosfat (pH 6) 10%, biotin: $4 \times 10^{-5}$%, MeOH, $ddH_2O$ | 0.0029 | 7.61 | 0.0030 |

*TES: Trace Element Solution, VS: Vitamin Solution, Me: Microelemet

### 3.4. Scale-up in 5-L Bioreactors

The recombinant HPV-52 L1 protein was expressed successfully and effectively, as evidenced by visualizations via SDS-PAGE and WB in the 5-L bioreactor (Figure 2). Continued optimization in a 5-L bioreactor with a 3-L working volume was essential to determine the optimal conditions for producing recombinant HPV-52 L1 protein. Through this series of experiments, we have successfully formulated a synthetic media recipe for the production of recombinant HPV-52 L1 protein within the *P. pastoris* expression system.

## 4. Discussion

The utilization of SYN6 media variations aimed to replace nutrient-rich media, which tend to incur high costs in large-scale production. Data in Table 1 indicate that the biomass levels produced are not necessarily in line with low or high L1 protein production in ELISA values. These findings suggest that the relationship between the expression of the recombinant HPV-52 L1 protein and the growth rate phase is non-linear, consistent with other studies on recombinant protein production in *P. pastoris* (Cunha *et al.* 2004; Schenk *et al.* 2008). Next, the ELISA values for SYN6 media, ranked from highest to lowest, were SYN6.10, SYN6.1, SYN6.6, SYN6.3, SYN6.8, SYN6.7, SYN6.9, and SYN6.11. Media BMMY exhibited a relatively high ELISA value compared to SYN6.1, SYN6.6, SYN6.3, SYN6.8, SYN6.7, SYN6.9, and SYN6.11. SYN6 medium consists of salts, vitamins, and trace elements that support high cell density growth, provided appropriate pH conditions for cell growth and a suitable carbon source are available (Jenzelewski 2002). We are sure that the use of SYN6 media is more cost-effective compared to nutrient-rich BMMY media. Besides, the optimal synthetic medium composition can maximize protein recombinant production.

SYN6.10 medium, previously unformulated by researchers, consists of salts [$(NH_4)_2SO_4$, KCl, $KH_2PO_4$, $MgSO_4 \cdot 7H_2O$, NaCl, $CaCl_2$], a trace element solution, a vitamin solution, microelements, a carbon source (glycerol, MeOH), and its nitrogen source is peptone. The difference in the composition of the carbon source between SYN6 and BMMY media affects protein production by *P. pastoris*. SYN6 contains an added glycerol, resulting in two carbon sources within it. Previous reports have indicated that *P. pastoris* can efficiently utilize both glycerol and MeOH as energy and carbon sources (Solà *et al.* 2007; Çelik *et al.* 2008; Jordà *et al.* 2014). In fact, the conventional promoters used for heterologous gene expression in this yeast (namely, PGAP, constitutive, and PAOX, inducible) have been isolated from genes related to glycerol and MeOH metabolism (Cos *et al.* 2006; Gasser *et al.*

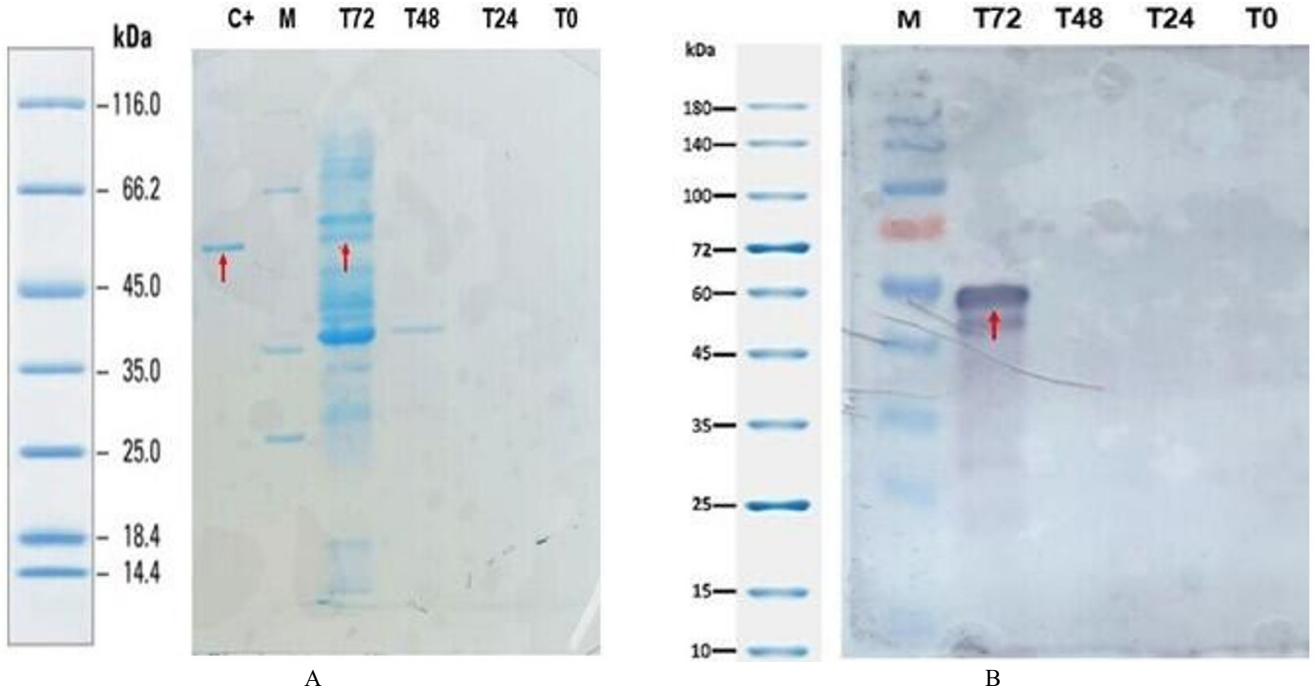



Figure 2. SDS-PAGE (A) and Western-blot 55 kDa shown by red arrow (B) in 5-L bioreactor

2013). Indeed, *P. pastoris* has been shown to grow on media containing crude glycerol (Lopes *et al.* 2013; Anastácio *et al.* 2014).

The comparison of growth profiles and biomass of *P. pastoris* is shown in Figure 3A and B. Following an adaptation period in all three types of media after 24 hours of fermentation, a relatively rapid growth phase occurred after 48 hours of fermentation, with no growth activity observed at 72 hours. The growth profile of *P. pastoris* corresponded to the biomass values produced, with optimal growth occurring at 48 hours, resulting in increased biomass values. Clearly, SYN6.10 media effectively promoted L1 production in *P. pastoris*, followed by BMMY and SYN6.1. The growth profile

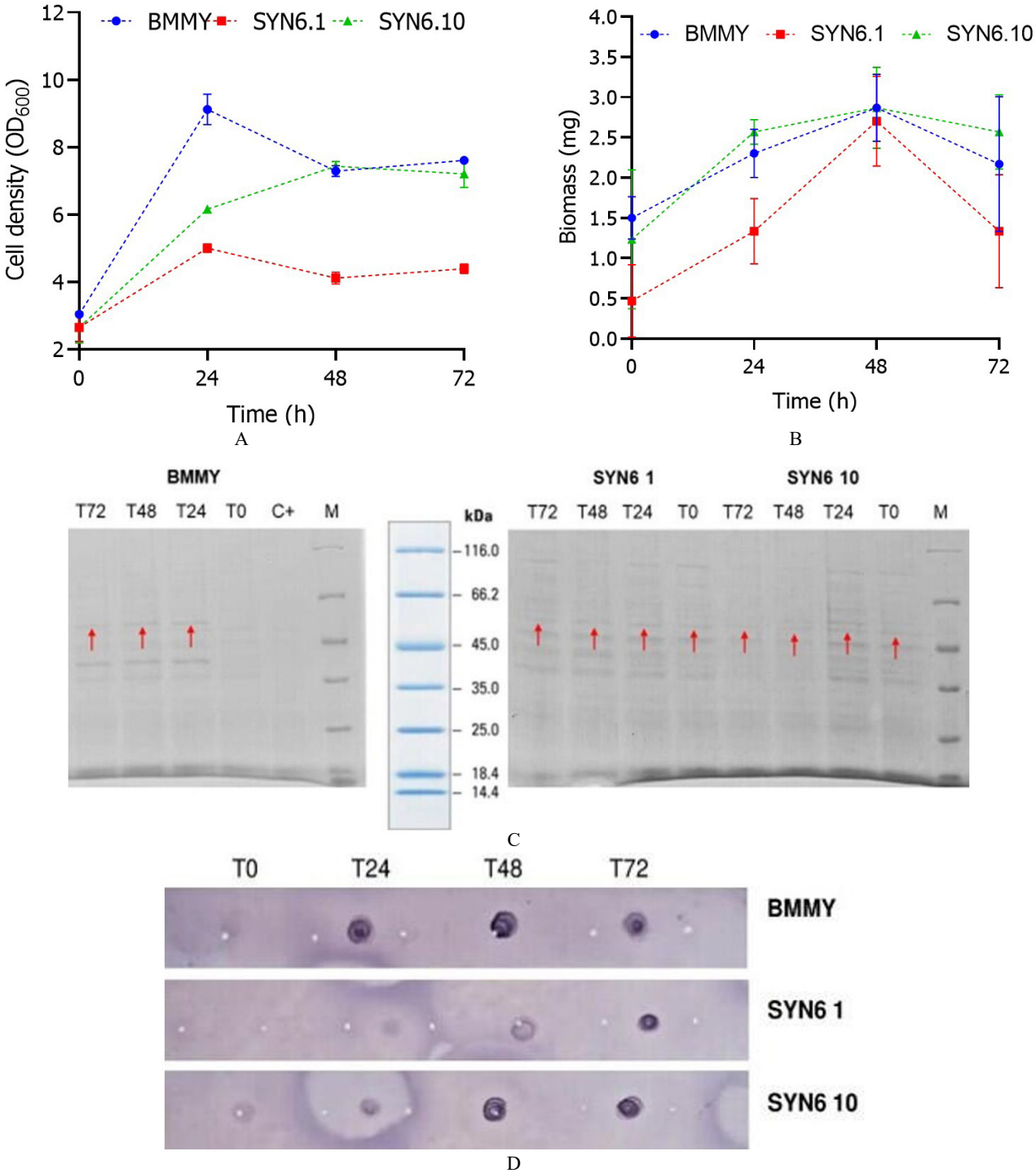



Figure 3. Three medium on mini bioreactor. (A) Growth profile, (B) Biomass, (C) SDS-PAGE 55 kDa shown red line, and (D) dot blot of *P. pastoris*

of *P. pastoris* using SYN6.10 was visually evident from the Dot-blot results (Figure 3D). In addition to glycerol, the carbon source added to SYN6.10 media was MeOH. Kongsinkaew *et al.* (2023) reported that the use of SYN6 synthetic media with the addition of the MeOH inducer can enhance peptide production. This is consistent with previous research reports, indicating that higher total methanol consumption by *P. pastoris* results in higher recombinant protein production (Damasceno *et al.* 2004; Wang *et al.* 2009; Charoenrat *et al.* 2015).

Bioreactors enable the production process to be conducted within optimal ranges of key bioprocess parameters (Panula-Perälä *et al.* 2008; Krause *et al.* 2010). Further experiments focused on using SYN6.10 media based on the results from the mini bioreactor scale. We optimized the modified media based on Mousa *et al.* (2012), particularly in terms of its composition and inducers, including $(NH_4)_2SO_4$: 7.66 g, KCl: 1.65 g, $KH_2PO_4$: 1 g, $MgSO_4 \cdot 7H_2O$: 3 g, NaCl: 0.15 g, enrichment [microelements: 5 ml, PTM1: 50 ml, 3% yeast extract: 30 ml, glycerol 50%: 40 ml, 2% peptone: 40 ml, CaCl: 20 ml], inducers [MeOH: 15%, peptone (2%): 20%, glycerol (50%): 22%, yeast extract (3%)], while eliminating the trace element solution and vitamin solution components. This modified medium is referred to as SYN6.10b in this work. The conditions for the 1-L bioreactor were as follows: agitation (400 rpm), temperature (30°C), pH (6.5), and DO maintained at 30%. In addition to the 1-L bioreactor, this experiment also compared the production of VLP L1 HPV 52 under mini bioreactor conditions: agitation (250 rpm), temperature (30°C), pH (5.5-6.5), DO (30%), and aeration at 3 vvm.

The data in Table 3 indicates that the highest biomass, $OD_{600}$, and ELISA values were obtained in the experiments using the 1-L bioreactor after 72 hours of fermentation. When observed in Figure 4, the relationship between cell growth rate and the produced cell mass demonstrates linear data. We suggest that this may be due to the higher growth rate resulting in greater biomass, which, in turn, leads to a higher presence of extracellular proteases in the cultivation. Bawa *et al.* (2014) stated that lower growth rates of *P. pastoris* cells also lead to lower biomass and the production of fewer extracellular proteases in the fermentation medium. Interestingly, the optimization data yielded different results compared to the previous mini bioreactor experiments using SYN6.10 media. The difference lies in whether or not MeOH inducer was added after fermentation. In the 1-L bioreactor experiment, after 24 and 48 hours of fermentation, 0.3% MeOH was added. *P. pastoris* cells were fermented for 24 hours to achieve a denser cell population, after which 0.3% MeOH was introduced. The reason for adding MeOH is to minimize the reduction in the expression of recombinant L1 protein due to the presence of glycerol during the batch phase.

Furthermore, Vogl and Glieder's (2013) stated that the pAOX1 promoter induces expression in the presence of MeOH, while glycerol or glucose fully represses it. The pAOX1 promoter has been the most commonly used promoter for heterologous protein expression in *P. pastoris* (Gasser *et al.* 2015). High-level expression of recombinant proteins has been consistently achieved with this promoter in *P. pastoris* (Hao *et al.* 2013; Chen *et al.* 2017). Similar reports by Charoenrat *et al.* (2015) and Wang *et al.* (2009) indicated that higher cell concentration before the MeOH induction step resulted in increased recombinant protein production. These results were attributed to the limited space in high-cell-density cultivation. With a high initial cell concentration, a lower percentage of MeOH was assimilated into biomass, while MeOH was oxidized to provide the necessary energy for recombinant protein production (Wang *et al.* 2009).

The results presented in Figure 5 depict the differences in cell growth and biomass profiles between the two types of bioreactors. Both the OD and biomass values were the highest in the 1-L bioreactor. This could be attributed to the larger quantity of nutrients and the larger reactor volume in the 1-L bioreactor, leading to higher cell density growth at the 1-L bioreactor scale. The SDS-PAGE and WB visualization, which was quite clear at 72 hours of fermentation, indicated the optimal fermentation time (Figure 4). For comparison, SYN6.10 medium, which only used 1% methanol as the inducer in the 1-L bioreactor at day 0 of fermentation (data not shown), resulted in a biomass value of 0.0031 g/L and an $OD_{600}$ value of 7.415, both of which were

Table 3. Productivity of *P. pastoris* at 72-h fermentation

| Biomass (g) | | $OD_{600}$ | | ELISA (µg/µL) | |
|---|---|---|---|---|---|
| Mini bioreactor | 1 L bioreactor | Mini bioreactor | 1 L bioreactor | Mini bioreactor | 1 L bioreactor |
| 0.0056 | 0.0124 | 11.20 | 21.60 | 0.15 | 0.24 |

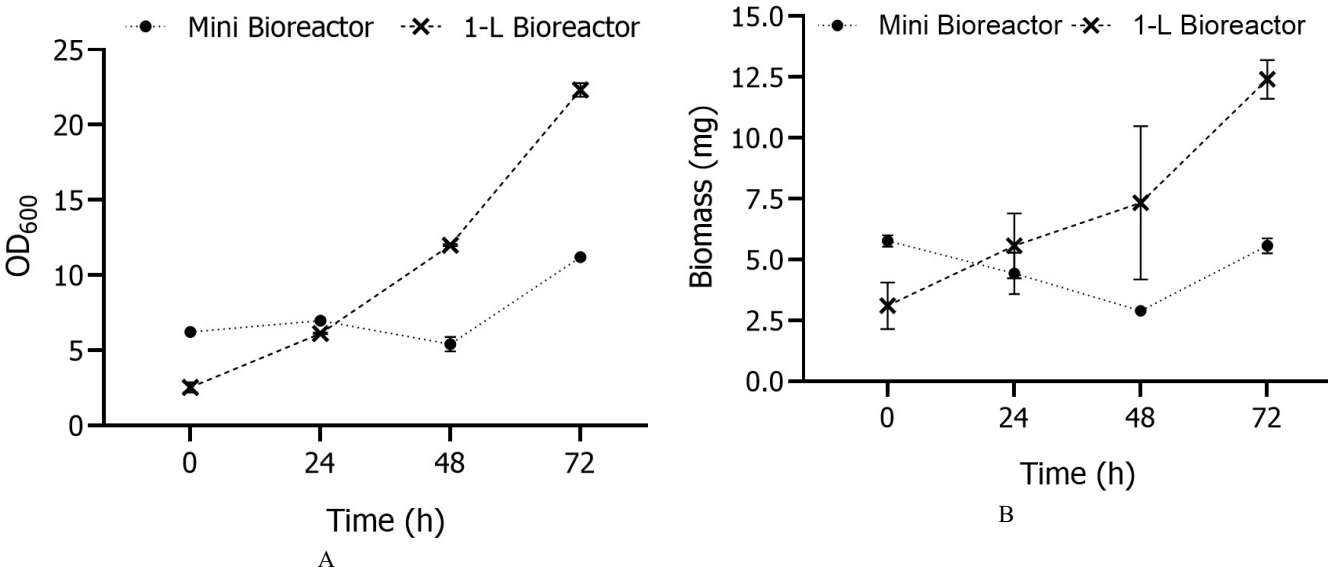



Figure 4. Cell density (A) and biomass (B) of *P. pastoris* grown in two different systems

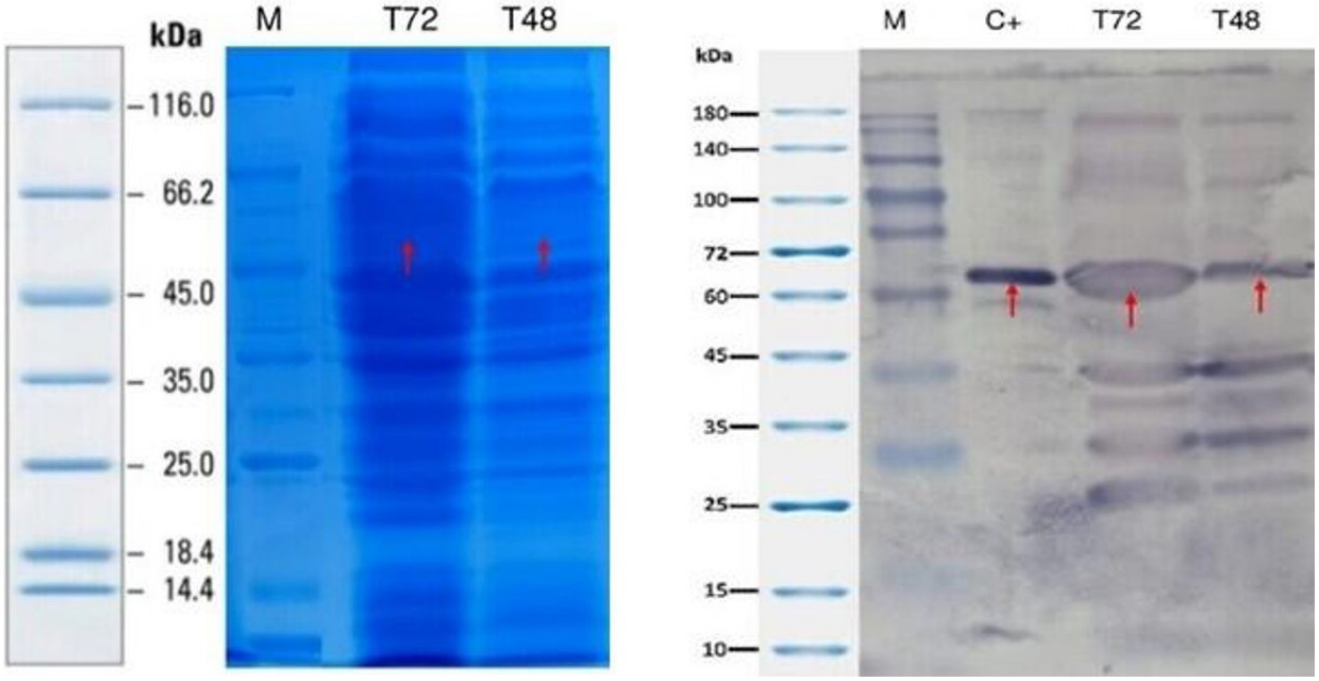



Figure 5. SDS-PAGE (A) and Western blot 55 kDa shown by red arrow (B) in 1-L bioreactor

smaller than those obtained with SYN6.10b medium. This suggests that the SYN6.10b medium is suitable for recombinant L1 protein production on a larger scale.

The culture was grown in SYN6.10b medium under the following conditions: agitation at 400 rpm, temperature at 30°C, pH at 6.5, and DO at 30%. Additionally, 0.3% MeOH (50%) was added as an inducer after 24 and 48 hours of fermentation. It is well-established that both the concentration and rate of MeOH addition to *P. pastoris* cultures can significantly impact recombinant protein yields (Çelik *et al.* 2009; Çelik *et al.* 2010; Potvin *et al.* 2012). The biomass and ELISA values in the 5-L bioreactor were 0.0068 g/L and 0.069 µg/100 µL, respectively (data not shown). The biomass yield of *P. pastoris* in a 5-L bioreactor volume with a 3L working volume was lower than that obtained in the 1-L bioreactor and the mini bioreactor. This phenomenon could be attributed to protein burden. Kafri *et al.* (2016) reported that protein overexpression

can reduce growth rates. Highly abundant transcripts of the recombinant protein compete for the limited ribosomal resources present in the host, which are otherwise responsible for translating endogenous proteins.

Out of a total of 17 media variations tested, we identified three distinct media formulations on a mini bioreactor scale, namely SYN6.1, SYN6.10, and BMMY. Ultimately, in the 1-L and 5-L bioreactor experiments, we employed a modified synthetic medium derived from SYN6.10, which we refer to as SYN6.10b. Our study introduces the first-ever report of a modified classic synthetic medium named SYN6.10b. This medium was applied during the glycerol batch phase, followed by the MeOH-fed batch phase, with the addition of 0.3% MeOH after 24 and 48 hours.

In conclusion, the findings of this study suggest that the modified SYN6.10b medium may serve as a viable alternative to the BMMY medium for the production of L1 HPV 52 recombinant protein using the *P. pastoris* expression system, with the potential to reduce overall production costs. Nevertheless, additional research is required to assess its effectiveness and efficiency thoroughly.

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