Research article

Comparing the expression of human DNA topoisomerase I in KM71H and X33 strains of *Pichia pastoris*

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ABSTRACT

Background: Human is an essential cellular enzyme that is found in all human cells. As this enzyme is upregulated in cancer cells exceedingly, it is used as a target for cancer chemotherapeutic drug development. As such, producing the in-house enzyme for the purpose to speed up the search for more cost-effective and target specific hTopoI inhibitors is warranted. This study aims to compare the optimised conditions for the expression of hTopoI in KM71H (Mut^S) and X33 (Mut$^+$) strains of *Pichia pastoris*. *P. pastoris* transfected with an hTopoI recombinant vector was used for the optimization of a higher level of hTopoI expression.
Results: In the process, fed-batch cultivation parameters that influence the expression of hTopoI, such as culture temperature, methanol induction and feeding strategy, were optimised in the transfected KM71H and X33 *P. pastoris* strains in a shake flask system. The cell density and total protein concentration (protein level) of transfected *P. pastoris* were compared to determine the optimum culture conditions for each transfected *P. pastoris* strain. A higher hTopoI level was observed in the transfected KM71H culture supernatant (2.26 ng/mL) when the culture was incubated in the optimum conditions.
Conclusions: This study demonstrated that Mut^S strain (KM71H) expressed and secreted a higher level of hTopoI heterologous protein in the presence of methanol compared to the Mut$^+$ strain; X33 (0.75 ng/mL). However, other aspects of optimization, such as pH, should also be considered in the future, to obtain the optimum expression level of hTopoI in *P. pastoris*.

1. Introduction

Human DNA topoisomerase I (hTopoI) is an essential cellular enzyme in all living cells, including cancers [1]. The enzyme regulates the topological state of DNA during critical cellular processes, such as DNA replication, gene transcription, recombination and repair, by transiently breaking one or two strands of DNA, passing single- or double-stranded DNA through the break and, finally resealing the DNA break [2,3]. The enzyme is often upregulated in cancer cells. It is a target for chemotherapeutic drugs in the Camptothecin (CPT) family [4,5], which are mainly used in the systemic treatment of colon, ovarian and small cell lung cancers (SCLC) [6,7].

The hTopoI regulates many events during the transcription cycle [8], whereby the cycle involves an intermediate covalent with the enzyme bound to the DNA complex (hTopoI-DNA-cc). This phenomenon allows the controlled rotation of the cleave DNA strand around the intact one. The cleavage cycle ends with the rejoin of the two extremities. The intermediate clevage reaction is exploited by cytotoxic agents, which have important applications as antibiotic agents and anticancer drugs [9]. The stabilization of the intermediate covalent by drugs, oxidative lesions or other agents results in the formation of a single DNA strand break that can arrest transcription or replication when the enzyme collides with an RNA polymerase or a DNA polymerase, respectively. In the latter case, the single DNA strand break is converted into a double DNA strand break. This phenomenon generates additional DNA break for the cells. The accumulation of DNA strand break overwhelms the cell and triggers the apoptotic mechanism in the cells [10]. The cytotoxicity of Camptothecin is triggered by trapping the drug to the hTopoI-DNA-cc. Two Camptothecin derivatives; topotecan and irinotecan are currently the only clinically approved hTopoI inhibitors [11]. In adults, topotecan is prescribed for ovarian and NSCLC, whereas irinotecan for colorectal cancers and both drugs are also effective in paediatric tumours. The broad clinical activity of topotecan and irinotecan in different cancers validates the importance of hTopoI as a therapeutic-based anticancer target. Novel hTopoI inhibitors are the focus of industry and academic research now [10,12]. Therefore, the production of the in-house enzyme to facilitate the continuous searching for more effective, affordable and target-specific hTopoI inhibitors as Camptothecin but cause fewer side effects, is necessary.

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This research aimed to produce a sufficient amount of in-house hTopoI to speed up screening of the enzyme inhibitors as potential anticancer agents from natural resources in our country.

The *Pichia pastoris* expression system has been widely used to produce a variety of different heterologous proteins, including hTopoI [13]. However, a comparison of production with different yeast strains has seldom been made. *P. pastoris* secretes very low levels of native proteins, making the foreign secreted protein is easier to recover from the fermentation fluid with a simple removal of the whole host cells by filtration or centrifugation. This project aimed to compare the optimum conditions of hTopoI expression in Mut^S strain (KM71H) and Mut$^+$ strain (X33) of *P. pastoris* transfected with the recombinant vector for hTopoI, to obtain a higher expression of the targeted protein. The X33 is a wild-type and the KM71H is a mutator strain of *P. pastoris*. The wild-type of *P. pastoris* has two alcohol oxidase genes; *AOX1* and *AOX2*, which are regulated by a strongly inducible promoter [14]. These genes allow *P. pastoris* to use methanol as carbon and energy sources. The targeted protein expression can be induced by the addition of methanol. The KM71H metabolises methanol poorly with very low oxygen consumption because the chromosomal *AOX1* gene of KM71H is largely deleted and replaced with the *Saccharomyces cerevisiae ARG4* gene [15]. As a result, this yeast strain relies on the much weaker *AOX2* gene, instead of *AOX1* gene, to grow on methanol. Unlike the KM71, the selection of KM71H can be carried out using Zeocin™ resistant expression vector (Table 1), which is synchronised with another yeast strain used in this study, X-33. This study may provide useful information on the optimum conditions for the *P. pastoris*, to speed up the hTopoI-targeting inhibitor screening from natural resources and synthetic drugs that are available in the country.

2. Materials and methods

2.1. Cultivation of different P. pastoris strains

P. pastoris strains KM71H (Mut^S) and X33 (Mut$^+$) were transformed with recombinant pPICZαA-hTopoI vector (5891 bp), to produce KM71H/pPICZαA-hTopoI and X33/pPICZαA-hTopoI. The recombinant vector was constructed from our previous study. It was originated from the vector pPICZαA (3593 bp, EasySelect™ *P. pastoris* Expression Kit, Cat. No: K1740-01, Invitrogen, USA). The yeast strains transformed with the pPICZαA vector without the inserted gene KM71H/pPICZαA and X33/pPICZαA were used as the background controls. All clones were grown on Yeast Peptone Dextrose agar plates that contained Zeocin. A single colony of each clone was inoculated into a universal bottle that contained 5 mL of buffered glycerol-complex medium (BMGY). The inoculums of transformed yeast and background controls were incubated at 28°C overnight in an incubator shaker (Thermo Scientific, USA) with the agitation speed of 250 rpm. After that, 250 μL of the overnight cultures were added to 250 mL conical flasks that contained 25 mL of BMGY (ratio 1:100). The cultures were shaken at 28°C and 250 rpm for another 16 h.

2.2. Culture induction stage

After 16 h of cultivation, the culture of each yeast strain was subjected to the optimization of culture temperature, induction reagent and feeding strategy. The optical density of the cultures was measured at 600 nm using a UV–Vis spectrophotometer (Thermo Scientific, USA) for cell density, whereas the concentration of total protein was measured at 595 nm using a dye-binding based method of the Bradford assay. The cultures were poured into 50 mL Falcon tube and centrifuged (Eppendorf, USA) at 4200 rpm for 5 min at 4°C. The supernatants were discarded for total protein concentration measurement, and the cell pellet was resuspended in 25 mL of buffered methanol-complex medium (BMMY) for cell density measurement. The centrifugation and resuspension were repeated once to remove the residual of BMGY in the cell pellet completely. Then, the suspensions were diluted to an OD_{600} (optical density at 600 nm) of 1 to a final volume of 25 mL with the BMMY in a 250 mL conical flask. The experiments were carried out in triplicate for all yeast strains. One (1) mL of the culture was collected from each flask at 12 h intervals (12, 24, 36, 48, 60 and 72 h) for measurement of cell density and total protein concentration, respectively. For every withdrawal, 1 mL of fresh BMMY was added to replace the withdrawn volume.

2.2.1. Optimization of culture temperature

Incubation of cultures was carried out in an incubator shaker (Thermo Scientific, USA) with the agitation speed of 250 rpm, at different temperatures, namely 15°C, 20°C, 25°C and 28°C. A 125 μL of methanol in 25 mL broth [0.5% (v/v)] was added to the cultures every 24 h. The cell density and total protein concentration of cultures grown at different temperatures were studied, to determine the optimal temperature for the yeast growth profile and total protein accumulated.

2.2.2. Optimization of induction reagent

After the optimal culture temperature of each yeast strain was determined, *i.e.*, 20°C, the cultures were incubated at the optimal temperature. The cultures were then induced with 25 μL and 250 μL of 100% (v/v) methanol in 25 mL broth, to produce final concentrations of 0.1% (v/v) and 1.0% (v/v) of methanol in the cultures, respectively. The agitation speed of the cultures was maintained at 250 rpm.

2.2.3. Optimization of feeding strategy

After the optimal concentration of methanol for induction was determined, *i.e.*, 1.0% (v/v), the optimal feeding strategy was determined by changing the frequency of the addition of methanol to the cultures. A 250 μL of 100% (v/v) methanol or 1.0% (v/v) of methanol in 25 mL broth was fed to the cultures every 12 h. The agitation speed of the cultures was maintained at 250 rpm.

2.3. Analytical measurements

2.3.1. Measurement of cell density

The growth profile of *P. pastoris* was analysed by measuring the cell density using UV–Vis spectrophotometer (Thermo Scientific, USA), as described in Section 2.2. The optical density of samples collected at 12, 24, 36, 48, 60 and 72 h was measured at 600 nm. The BMMY was used as blank in the measurement.

Table 1
The general information of KM71H and X33 strains of *P. pastoris*.

Strain	Genotype	Phenotype	Application
KM71H	*arg4 his4 aox1::ARG4*	Mut^S, Arg +	Selection of Zeocin™ resistant expression vector to generate yeast strain with Mut^S phenotype.
X33	Wild-type	Mut$^+$	Selection of Zeocin™ resistant expression vector to generate yeast strain with Mut$^+$ phenotype.

2.3.2. Measurement of total protein concentration

The supernatants collected in Section 2.2 were centrifuged again at 10,000 rpm, 4°C for 10 min (Eppendorf, USA). The supernatant was used for the determination of total protein concentration by the Bradford assay using a microplate reader (TECAN Sunrise, Switzerland), at 595 nm. The bovine serum albumin (BSA) was used as the standard protein of a curve for this measurement. The total protein concentration of each sample was then estimated from the constructed standard curve.

2.4. Measurement of hTopoI concentration

The hTopoI concentration was measured using Cloud-Clone Corp. Enzyme-Linked Immunosorbent Assay (ELISA) kit for Topoisomerase I (TOP1). For this, 100 μL of the standard or sample was added to appropriate wells of a pre-coated 96-wells strip plate. The plate was then incubated at 37°C for 2 h. The solution in the wells was aspirated, and 100 μL of diluted Detection Reagent A was added to the wells. The plate was incubated again at 37°C for 1 h. The solution in the wells was aspirated again before washed twice with 350 μL of washing buffer. The washing buffer was pipetted and allowed to remain in each well for 1–2 min for each washing step. After the washing, 100 μL of diluted Detection Reagent B was added to the wells. The plate was incubated at 37°C for 30 min. The solution in the wells was aspirated and washed with 350 μL washing buffer 5 times. The washing buffer in the wells was pipetted and remained for 1–2 min again for each washing step. A 90 μL of substrate solution was then added to the wells followed by incubation at 37°C for 20 min. Once the substrate solution was added into the wells, the solution turned bluish. The solution turned yellow after 50 μL of stop solution was added to the wells. The developed colour in the plate was immediately read at 450 nm by a microplate reader (TECAN Sunrise, Switzerland).

2.5. Measurement of hTopoI activity

For hTopoI activity measurement, a total volume of 20 μL reaction system was prepared. The reaction solution comprised of 4 μL of 5× hTopoI reaction buffer, 0.25 μg supercoiled pBR322 (Inspiralis, UK) and 1 μL of supernatant of each culture. The culture supernatant contained recombination enzyme expressed by KM71H/pPICZαA-hTopoI or X33/pPICZαA-hTopoI at optimised conditions for 12, 24, 36, 48, 60, 72, 84 and 96 h. The reaction was incubated at 37°C for 30 min and then terminated by adding 4 μL of stop reaction buffer (6×). The samples were electrophoresed (Bio-Rad, USA) in 1% (w/v)

agarose gel and the gel was stained with ethidium bromide for 5 min. The gel was destained in dH_2O and photographed under UV with Alpha Innotech Fluorchem FC2 (Fisher Scientific, Canada).

2.6. Statistical analysis

All graphs and all statistical calculations were generated and performed using *GraphPad* 6.05 software (GraphPad Software, USA). The experiments were repeated several times to confirm the reproducibility of the results. All values were expressed as the mean ± SD. A *p* value less than 0.05 was considered as statistically significant.

3. Results

3.1. The optimization of culture temperature

The growth profile (cell density) and concentration of total protein (protein level) of the transformed yeast incubated at different culture temperatures and incubation periods were compared in this study. In brief, incubations of all transformed *P. pastoris* strains; KM71H/pPICZαA-hTopoI and X33/pPICZαA-hTopoI were first carried out at 15°C, 20°C, 25°C and 28°C, and shaken at 250 rpm. The yeast cultures were fed with 0.5% (v/v) of methanol every 24 h.

The KM71H/pPICZαA-hTopoI showed different growth profiles and total protein concentrations under various culture conditions. As shown in Fig. 1a, the growth of KM71H/pPICZαA-hTopoI showed the highest cell density at 20°C after 60 h of cultivation. The cell density at this incubation period was (11.48 ± 0.080) unit ($p < 0.01$), increased by approximately 56.6% compared to the cell density of same transformed yeast incubated at the same culture temperature for 12 h; (7.33 ± 0.060) unit. The total protein concentration of KM71H/pPICZαA-hTopoI was also the highest at 20°C, incubated for 72 h (0.10 ± 0.002) μg/μL ($p < 0.05$), Fig. 1b. The total protein concentration of the transformed yeast increased by approximately 100.0% compared to the total protein concentration of the same transformed yeast cultivated at the same culture temperature for 12 h; (0.05 ± 0.009) μg/μL. The optimal conditions for KM71H/pPICZαA-hTopoI cultivation were at 20°C for at least 60 h, and these culture conditions were used for subsequent experiments. This phenomenon also indicated that a prolonged incubation period was required for maximum growth and protein expression in the KM71H strain.

As shown in Fig. 2a, the growth of X33/pPICZαA-hTopoI was the highest, (11.12 ± 0.040) unit ($p < 0.05$), at the culture temperature of
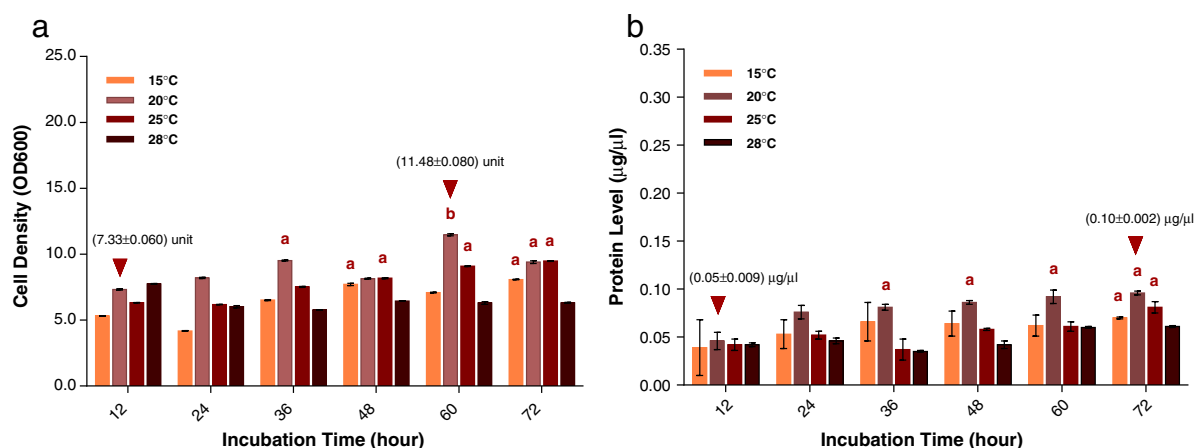

Fig. 1. (a) Growth and (b): protein expression of KM71H/pPICZαA-hTopoI at different culture temperatures and incubation periods. The cultures were incubated at 15°C, 20°C, 25°C and 28°C, with 0.5% (v/v) of methanol feeding every 24 h. "a" and "b" represent $p < 0.05$ and $p < 0.01$, respectively, compared with the value and level at 12 h.
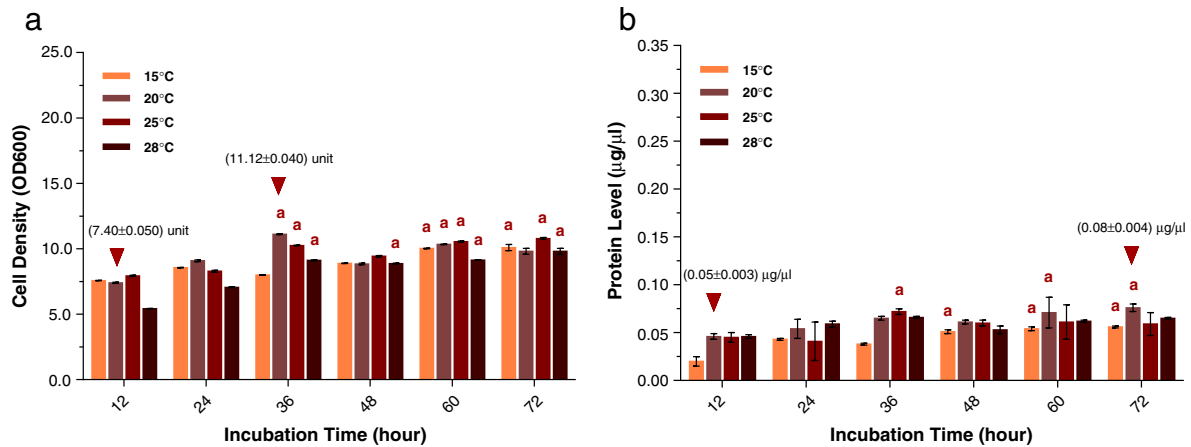
Fig. 2. (a) Growth and (b): protein expression of X33/pPICZαA-hTopoI at different culture temperatures and incubation periods. The cultures were incubated at 15°C, 20°C, 25°C and 28°C, with 0.5% (v/v) of methanol feeding every 24 h. "a" represents $p < 0.05$, compared with the value and level at 12 h.

20°C after 36 h of cultivation. The cell density of the transformed yeast after 36 h of cultivation was increased by approximately 50.3% compared to the cell density of same transformed yeast cultivated at the same temperature for 12 h, (7.40 ± 0.050) unit. On the other hand, the total protein concentration of X33/pPICZαA-hTopoI was the highest at 20°C for the transformed yeast incubated for 72 h (0.08 ± 0.004) μg/μL ($p < 0.05$), Fig. 2b. The total protein concentration at 20°C of this incubation period increased by approximately 60.0% compared to the total protein concentration of the transformed yeast incubated at the same culture temperature for 12 h; (0.05 ± 0.003) μg/μL. Thus, the optimal culture condition for X33/pPICZαA-hTopoI was at 20°C for at least 36 h, and this culture condition was used for subsequent experiments. The optimization of culture temperature also indicates that both transformed yeast reached maximum growth and protein expression at 20°C.

The transformed KM71H strain required a prolong incubation time (≥60 h) to reach the maximum growth and protein expression, compared to the transformed X33 strain (≥36 h). Although the maximum levels of cell density and protein of both transformed strains were identified, however, only the KM71H/pPICZαA-hTopoI cultivated at 20°C for 60 h showed significant increase on the cell density. No apparent changes on the cell density of X33/pPICZαA-hTopoI and the protein level of both transformed strains were observed throughout the experiments.

3.2. The optimization of induction reagent

At the identified optimum culture temperature and incubation period determined in the previous section, the transformed *P. pastoris* were fed with absolute methanol at final concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) every 24 h. The agitation speed of the cultures was set at 250 rpm for all cultures.

At 20°C, the KM71H/pPICZαA-hTopoI showed the highest cell density when the culture was fed with 1.0% (v/v) of methanol every 24 h for 72 h; (14.59 ± 0.090) unit ($p < 0.001$), Fig. 3a. The cell density at this incubation period was increased by approximately 135.7%, compared to the cell density of the same transformed cells incubated for 12 h; (6.19 ± 0.050) unit, under the same culture conditions. Moreover, the total protein concentration of KM71H/pPICZαA-hTopoI was also the highest when the culture was fed with 1.0% (v/v) of methanol every 24 h for 60 h; (0.11 ± 0.023) μg/μL ($p < 0.01$), Fig. 3b. The protein expressed under this culture condition was increased by approximately 175.0%, compared to the total protein concentration of the same transformed cells at 12 h; (0.04 ± 0.005) μg/μL, under the same culture conditions. Therefore, 1.0% (v/v) of methanol is the optimum concentration of induction reagent for the KM71H/pPICZαA-hTopoI, to induce the highest concentration of total protein in the transformed yeast.
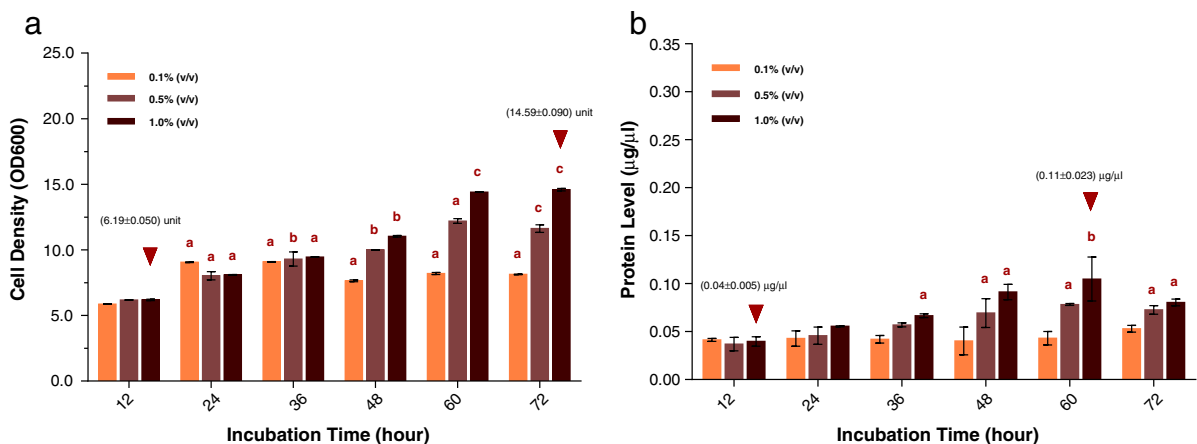

Fig. 3. (a) Growth and (b): protein expression of KM71H/pPICZαA-hTopoI at different incubation time-points. The cultures were incubated at 20°C and fed with 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) of methanol, every 24 h. "a", "b" and "c" represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared with the value and level at 12 h.
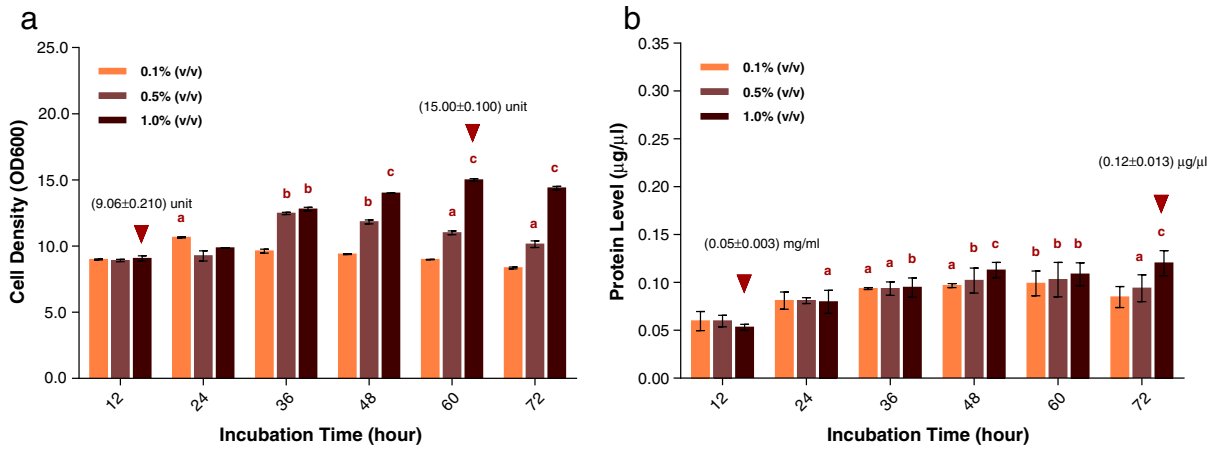
Fig. 4. (a): Growth and (b): protein expression of X33/pPICZαA-hTopoI at different incubation time-points. The cultures were incubated at 20°C and fed with 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) of methanol, every 24 h. "a", "b" and "c" represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared with the value and level at 12 h.

As shown in Fig. 4a, X33/pPICZαA-hTopoI exhibited the highest cell density when the culture was fed with 1.0% (v/v) of methanol every 24 h for 60 h; (15.00 ± 0.100) unit ($p < 0.001$), at 20°C. The cell density at this incubation period was increased approximately 65.6%, compared to the cell density of the same transformed yeast incubated for 12 h; (9.06 ± 0.210) unit, under the same culture conditions. Moreover, the protein expressed by the X33/pPICZαA-hTopoI was the highest when the culture was fed with 1.0% (v/v) of methanol every 24 h for 72 h; (0.12 ± 0.013) μg/μL ($p < 0.001$), Fig. 4b. The total protein concentration in this culture condition was increased by approximately 140.0%, compared to the total protein concentration of the same transformed culture incubated for 12 h; (0.05 ± 0.003) μg/μL, under the same culture conditions. Therefore, 1.0% (v/v) of methanol is the optimum concentration of induction reagent for maximum protein expression in the X33/pPICZαA-hTopoI.

In brief, X33/pPICZαA-hTopoI required a shorter incubation period to produce the highest level of cell density, but longer period to produce the highest level of protein expression, compared to the KM71H/pPICZαA-hTopoI. However, KM71/pPICZαA-hTopoI may produce a similar level of cell density at 60 h and 72 h of cultivation. Therefore, the high level of cell density can also be obtained at 60 h of cultivation for both transformed yeasts that fed with absolute methanol at final concentration of 1.0% (v/v).

3.3. The optimization of feeding strategy

The optimum culture temperature and concentration of induction reagent determined in the previous section were then used for feeding strategy optimization in which the KM71H/pPICZαA-hTopoI and the X33/pPICZαA-hTopoI were incubated at 20°C with 1.0% (v/v) of methanol feeding every 12 h and every 24 h for 72 h. The agitation speed of the cultures was maintained at 250 rpm during all incubations. The cell density and total protein concentration of each culture were compared every 12 h and 24 h.

Both KM71H/pPICZαA-hTopoI and X33/pPICZαA-hTopoI showed the highest levels of cell density and total protein concentration when the cultures were fed with 1.0 (v/v) of methanol every 12 h for 72 h (Fig. 5, Fig. 6). The cell density and total protein concentration in KM71H/pPICZαA-hTopoI at 72 h of cultivation were (19.52 ± 1.000) unit ($p < 0.001$) and (0.14 ± 0.002) μg/μL ($p < 0.001$), respectively. The cell density at this incubation period has increased approximately 244.3%, compared to the cell density of the same transformed yeast incubated for 12 h; (5.67 ± 0.010) unit, under the same culture conditions, whereas the total protein concentration at this incubation period has increased approximately 600.0%, compared to the level of the same transformed yeast incubated for 12 h; (0.02 ± 0.001) μg/μL, under the same culture conditions.
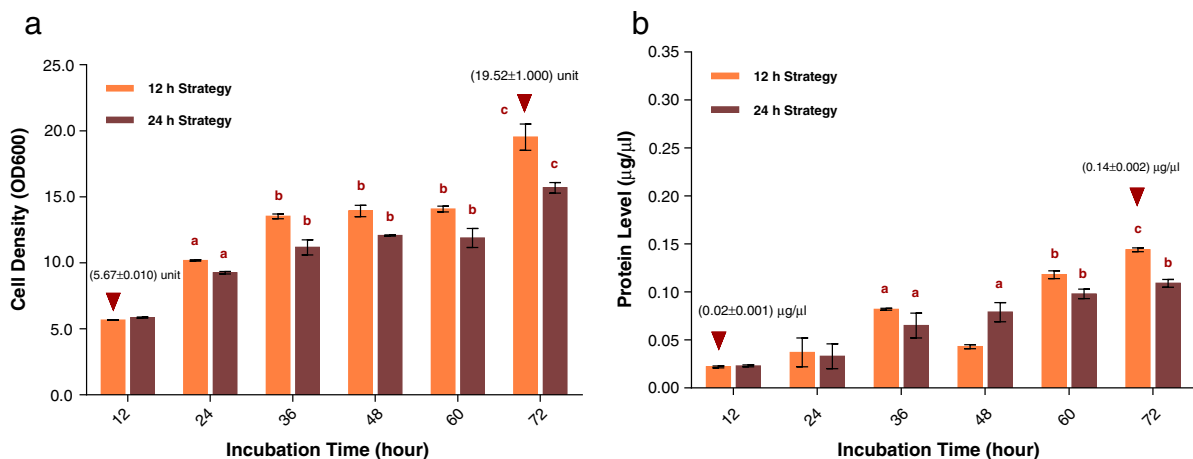

Fig. 5. (a): Growth and (b): protein expression of KM71H/pPICZαA-hTopoI at different incubation time points. The cultures were incubated at 20°C with 1.0% (v/v) methanol concentration feeding every 12 h and 24 h. "a", "b" and "c" represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared with the value and level at 12 h.
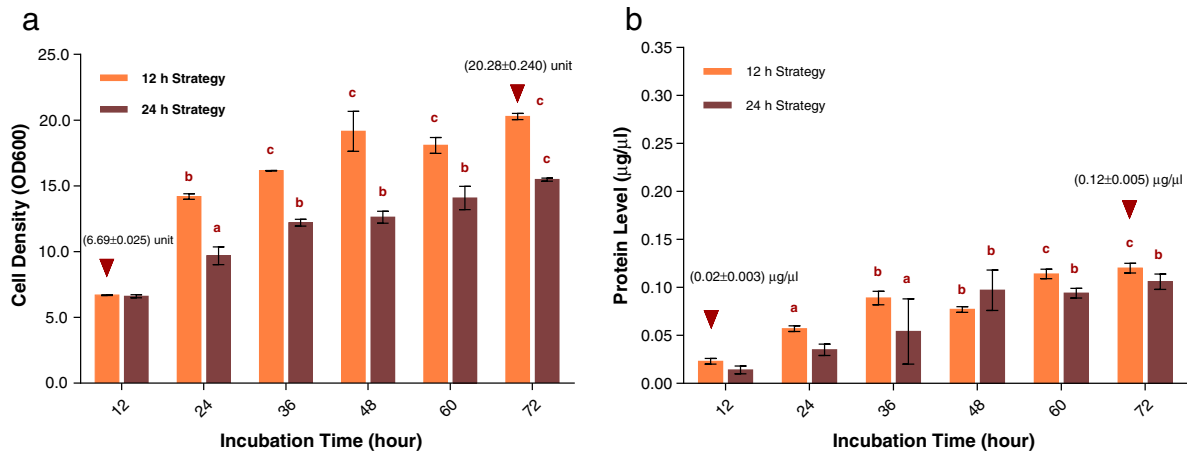
Fig. 6. (a): Growth and (b): protein expression of X33/pPICZαA-hTopoI at different incubation time points. The cultures were incubated at 20°C with 1.0% (v/v) methanol concentration feeding every 12 h and 24 h. "a", "b" and "c" represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared with the value and level at 12 h.

On the other hand, the cell density and total protein concentration in X33/pPICZαA-hTopoI were (20.28 ± 0.240) unit $(p < 0.001)$ and (0.12 ± 0.005) µg/µL $(p < 0.001)$, respectively, at the culture conditions as mentioned above. The cell density at this incubation period has increased approximately 203.1%, compared to the cell density of the same transformed yeast incubated for 12 h; (6.69 ± 0.025) unit, under the same culture conditions, whereas the total protein concentration at this incubation period has increased approximately 500.0%, compared to the level of the same transformed yeast incubated for 12 h; (0.02 ± 0.003) µg/µL, under the same culture conditions. Methanol feeding every 24 h for 72 h also resulted in high level of cell density and total protein concentration in the transformed yeast strains, but the levels were not as high as when methanol was added every 12 h. Therefore, methanol feeding every 12 h was determined to be the optimal feeding strategy for the growth of KM71H/pPICZαA-hTopoI and X33/pPICZαA-hTopoI.

Although both transformed yeasts showed the highest levels of cell density and total protein concentration at 72 h of cultivation, a similar high level of cell density and protein level as the maximum level can also be obtained at 48 h and 60 h, respectively in the X33/pPICZαA-hTopoI, indicating that the X33/pPICZαA-hTopoI required a shorter incubation period to produce the highest level of cell density and protein level than the KM71H/pPICZαA-hTopoI.

3.4. The identification of hTopoI using ELISA

The ELISA was carried out to confirm the level of targeted protein expression in the transformed X33 and KM71H strains under the optimum conditions (Fig. 7). The KM71H/pPICZαA-hTopoI expressed high levels of hTopoI when the transformed yeast was incubated under the optimum conditions for 60 h (1.06 pg/µL) and 72 h (2.26 pg/µL), respectively. The levels of targeted protein were detected in the X33/pPICZαA-hTopoI as 0.50 pg/µL and 0.75 pg/µL, when the transformed yeast was incubated under the optimum conditions for 60 h and 72 h, respectively. Overall, the KM71H/pPICZαA-hTopoI expressed a higher level of hTopoI compared to the X33/pPICZαA-hTopoI at the same culture conditions and time points. The results of hTopoI optimization were also briefly summarized in Table 2.

3.5. The activity of enzyme in culture supernatants

The DNA relaxation assay revealed that the enzyme activity in the supernatants of KM71H-pPICZ-α-A-TopoI and X-33-pPICZ-α-A-TopoI was detected in all cultures, at all time points. The supercoiled pBR322 was fully nicked and relaxed in the reactions incubated with the culture supernatants of KM71H-pPICZ-α-A-TopoI collected at 48 h
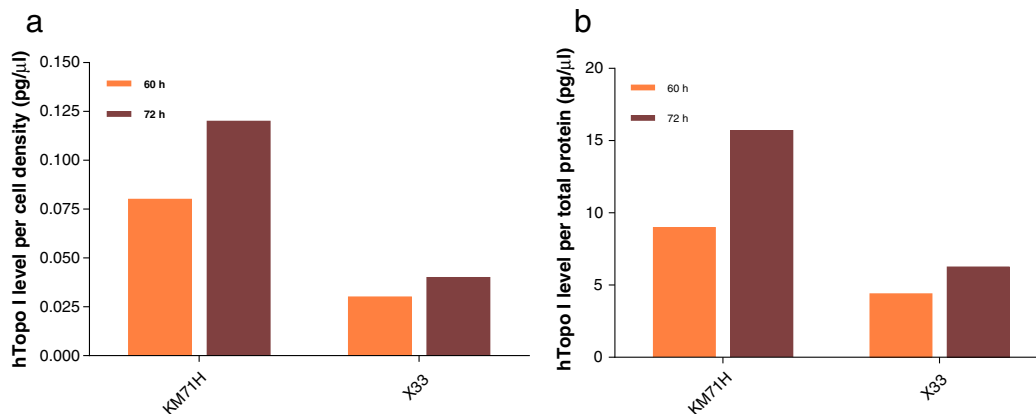

Fig. 7. (a): The level of hTopoI (pg/µL) in every unit of OD absorbance at 600 nm; which is equivalent to 1.4×10^7 cells/mL, and (b): in every µg/µL of total protein accumulated. The KM71H/pPICZαA-hTopoI and X33/pPICZαA-hTopoI were incubated at 20°C with 1.0% (v/v) methanol feeding every 12 h for 60 h and 72 h.

Table 2
Summary of the optimised culture temperature, concentration of methanol and feeding strategy on the cell density and protein level of the transformed KM71H and X33 yeast strains.

		KM71H	X33
Culture temperature	Cell density	20°C, 60 h	20°C, 36 h
		(11.48 ± 0.080) unit	(11.12 ± 0.040) unit
	Total protein	20°C, 72 h	20°C, 72 h
		(0.10 ± 0.002) µg/µL	(0.08 ± 0.004) µg/µL
Induction reagent (Methanol)	Cell density	1.0% (v/v), 72 h	1.0% (v/v), 60 h
		(14.59 ± 0.090) unit	(15.00 ± 0.100) unit
	Total protein	1.0% (v/v), 60 h	1.0% (v/v), 72 h
		(0.11 ± 0.023) µg/µL	(0.12 ± 0.013) µg/µL
Induction strategy	Cell density	Every 12 h for 72 h	Every 12 h for 72 h
		(19.52 ± 1.000) unit	(20.28 ± 0.240) unit
	Total protein	Every 12 h for 72 h	Every 12 h for 72 h
		(0.14 ± 0.002) µg/µL	(0.12 ± 0.005) µg/µL

and onwards, indicating that the enzyme was significantly expressed in the culture supernatants of 48 h and onwards (Fig. 8a). On the other hand, the culture supernatants of X-33-pPICZ-α-A-TopoI collected at different time points also exhibited the enzyme activity as indicated by the production of nicked and relaxed supercoiled pBR322, as revealed by the DNA relaxation assay (Fig. 8b). The DNA relaxation assay showed that no enzyme activity was observed in the culture supernatants of KM71H-pPICZ-α-A and X-33-pPICZ-α-A in all reactions (data not shown).

4. Discussion

Our results demonstrate that both yeast strains exhibited optimum levels of cell density and total protein concentration at 20°C with 1.0% (v/v) of methanol feeding strategy every 12 h for 72 h. Although, the optimization improved the growth of both transformed yeast strains, it did not affect much on the concentration of total protein. Indeed, both yeast strains accumulated maximum concentration of total protein at 72 h, but only a higher level of hTopoI was detected in the KM71H/pPICZαA-hTopoI, compared to the X33/pPICZαA-hTopoI.

Low culture temperatures have shown to improve the solubility of heterologous proteins in E. coli [16,17,18,19], and low culture temperatures might benefit from better protein folding. Cultivating the cells at lower culture temperatures reduces the rate of protein synthesis and thus may allow more time for the nascent peptide chains to fold properly. It has been reported that intermolecular disulphide bonds are preferentially formed at higher temperatures

when the protein is expressed in E. coli [20,21]. The hTopoI is not an intermolecular and hence it is not affected by temperature. This phenomenon at least partially explains that the protein expression at 20°C is much higher than that of the protein expressed at 25°C and 28°C in both transformed yeast strains. Moreover, higher temperatures may also lead to exposure of more hydrophobic surfaces during peptide folding and favour hydrophobic interaction and thus may predispose hTopoI to aggregation. Misfolded and aggregated proteins are more susceptible to the intracellular proteolytic degradation [22,23]. Therefore, inducing protein expression at a lower temperature may help to reduce protein misfolding and allow more properly folded protein to be secreted into the culture medium.

Overall, the cell density of the transformed yeast cultured at 20°C was the highest compared to the transformed yeast cultured at 25°C and 28°C, probably also due to the stabilization of cell membrane at a lower temperature. This stabilization would reduce the amount of proteases released from dead cells and thus reduce the degradation of the secreted protein. In general, low culture temperature expression may be widely applicable to increase the yields of aggregation-prone and/or unstable gene products in P. pastoris. Moreover, a low culture temperature may improve heterologous protein production by reducing the proteolytic activity of the cells and hence reduce the cell lysis [24,25].

The cell density and concentration of secreted protein increase proportionally to the concentration of methanol in the culture medium. Some studies have shown that a lower methanol concentration in the induction phase reduces the cell growth rate, which consequently reduces the expression of the heterologous protein [13,26]. The methanol serves as the sole carbon source for metabolism in the bioprocess. Indeed, additional glycerol can be consumed before methanol feeding, to induce the AOX1 promoter and allow the yeast to grow on methanol fully. It has been reported that a "mixed feed" of glycerol and methanol successfully expresses recombinant proteins [27,28]. Moreover, it is important to introduce methanol slowly to adapt the culture to grow on methanol. Adding the methanol too quickly is harmful to the cells. Growing yeast cells on methanol also generate a great deal of heat, so temperature control at this stage is very important.

In this study, the KM71H (Mut^S strain) was observed to express a higher level of hTopoI than the X33 (Mut$^+$ strain). The Mut^S yeast strains, such as KM71H, metabolise methanol poorly with very low oxygen consumption. The chromosomal AOX1 gene of KM71H is largely deleted and replaced with the S. cerevisiae ARG4 gene [15]. As a result, this yeast strain relies on the much weaker AOX2 gene, instead of AOX1 gene, to grow on methanol. This is the reason that the KM71H is a slower rate of growth of yeast strain. However, unlike Mut$^+$ strain, Mut^S strain consumes a high amount of methanol, compared to the Mut$^+$ wild-type whose growth is inhibited due to the accumulation of formaldehyde and hydrogen peroxide until these metabolic products exceed the toxic limit. Additionally, formaldehyde
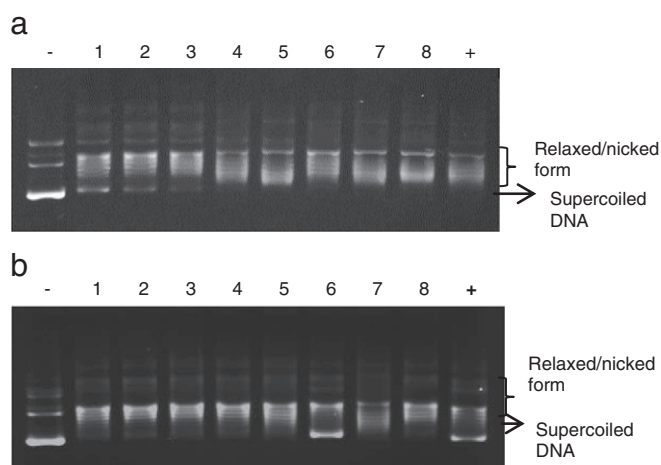

Fig. 8. The DNA relaxation assay for culture supernatants of (a) KM71H-pPICZ-α-A-TopoI and (b) X-33-pPICZ-α-A-TopoI. Lane (-): control negative without any enzyme. Lanes 1–8: 1 µL of culture supernatant collected at 12, 24, 36, 48, 60, 72, 84 and 96 h of cultivation. Lane (+): Positive control with 1 U of DNA Topoisomerase I enzyme (TopoGen, USA).

and hydrogen peroxide increase oxidative stress and compromise cell viability. As a consequence, lysed cells release proteases to degrade further the heterologous protein [13,29].

The quantification analysis of the expression of hTopoI under the optimal condition for each transformed *P. pastoris* strain by ELISA revealed that the KM71H expressed a higher level of the enzyme, compared to the X33. For every unit of OD absorbance at 600 nm; which is equivalent to 1.4×10^7 cells/mL, the hTopoI accumulated in the KM71H culture at 60 h and 72 h of cultivation were 0.08 pg/μL and 0.12 pg/μL, respectively, which were followed by the hTopoI accumulated in the X33 culture. The enzyme accumulated in the X33 culture at 60 h and 72 h of cultivation was 0.03 pg/μL and 0.04 pg/μL, respectively. In general, there were 8.98 pg (w/w) and 15.69 pg (w/w) of hTopoI accumulated in every μg of total protein accumulated in the KM71H culture at 60 h and 72 h of cultivation, respectively, and the hTopoI accumulated in the X33 culture at 60 h and 72 h of cultivation were 4.39 pg (w/w) and 6.25 (w/w), respectively. Therefore, the KM71H is a better yeast strain for the targeted enzyme expression than the X33.

It is suggested that in addition to temperature, methanol concentration and feeding strategy in the induction stage, other cultivation conditions, such as pH, addition of protease inhibitors, peptone, casamino acids or specific amino acids, changing medium composition (*e.g.*, carbon and nitrogen sources) can also be optimised [30,31,32]. A protease-deficient yeast strain, SMD1168h (*pep4*) is suggested to be used for the comparison with KM71H and X33 strains in the future study. The SMD1168h strain has been effectively used in heterologous protein production [33], because it is deficient in protease A, which then causes a deficiency in carboxypeptidase Y and protease B. As such, fewer proteases were released into the medium compared with the wild-type X33 strain. Therefore, less degradation of the hTopoI may occur in the SMD1168h compared to the X33. The use of alternative promoters instead of *AOX1* promoter is also suggested. The glutathione-dependent formaldehyde dehydrogenase (FLD) promoter was used successfully to drive the production of heterologous proteins. Nevertheless, the expression levels of the targeted proteins in glucose media are significantly higher than those obtained with *AOX1* promoter in the presence of methanol [34]. The *AOX2* promoter (full or truncated version) was also reported to drive moderate gene expression [35]. Although its expression level is much lower than *AOX1* promoter, Kobayashi et al. [36] demonstrated that the addition of small amounts of oleic acid (0.01%) led to a two-fold increased protein production due to the presence of an oleic acid-responsive element within the *AOX2* promoter.

5. Conclusions

The hTopoI was successfully expressed and secreted by the KM71H and the X33 strains of *P. pastoris* in which the temperature, methanol concentration and feeding strategy in the induction stage were optimised and also studied. The results proved that the hTopoI was better expressed by the KM71H strain, compared to the X33 strain based on the optimised conditions.

Conflict of interest

No conflicts of interest between the researchers in this topic of research.

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