Multiple gene copy number increases total protein expression and enzyme activity of DNA topoisomerase I in *Pichia pastoris*

NUR ADILA FADZIL, SHERN KWOK LIM, AI LAN CHEW and BOON YIN KHOO

Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia (USM), 11800 Penang, Malaysia

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Abstract. The present study aimed to employ an *in vivo* strategy for the establishment of multi-copy gene constructs of human DNA topoisomerase I (hTopI) using the pPIC3.5K vector in *Pichia*. The clones with multi-copy inserts (His^+ transformants) that were able to survive in the highest concentration of Geneticin® were found to express the highest expression level of total protein and exhibited the highest target enzyme activity. The highest level of total protein found was 1.76 mg/ml in GS115-pPIC3.5K-hTop1, which was resistant to 1.00 mg/ml Geneticin at 48 h of incubation. The highest enzyme activity of hTopI was also observed in the culture expressed by GS115-pPIC3.5K-hTopI, which was resistant to 1.00 mg/ml Geneticin® ($19.7x10^4$ Ul/OD_{600}). On the whole, the present study provides information regarding the production of target protein from recombinant *Pichia* using only a shaker flask system, which can be further developed as an in-house resource for screening potential anticancer agents.

Introduction

DNA topoisomerases play crucial roles in cellular processes by regulating the topological state of DNA (1). The inhibition of DNA topoisomerase activities is an approach used for screening anticancer agents. Agents that react to enzymes are predicted to inhibit the growth of cancer cells. Therefore, the enzymes can be exploited as scientific tools to rapidly screen novel inhibitors *in vitro* as anticancer agents (2). The use of multi-copy number gene strategies to express a large yield of specific enzymes, e.g., human DNA topoisomerase I (hTopI), increases the sensitivity of the host towards growth inhibitors. In a number of cases, the expression found using single-copy gene transformants has been disappointingly low. A strategy for constructing multi-copy numbers of genes that produces tandem inserts of a gene by ligation (*in vitro* strategy) has been utilized, and this was found to be one of the most effective strategies to increase gene expression (3). By contrast, a more popular *in vivo* strategy, which utilizes hyper-resistance to an antibiotic that allows users to screen the multi-copy inserts, conferring resistance to the antibiotic, has not yet been used to express hTopI. It is hypothesized that the transformation of *Pichia* with multi-copy number genes may increase cell density, total protein expression and enzyme activity in the host to increase the sensitivity of the host to growth inhibitor screening.

Pichia pastoris is a methylotrophic yeast and can be used as a more rapid and cost-effective eukaryotic protein expression system than baculovirus or mammalian tissue culture (3). The increasing popularity of the *Pichia* expression system is due to the availability of different strains commercially available as expression systems, the simplicity of the techniques needed for molecular genetic manipulation and the similarity to those of *Saccharomyces cerevisiae* (*S. cerevisiae*). *Pichia* produces intracellular and extracellular foreign proteins at high levels and undergoes a number of eukaryotic post-translational modifications, while being as easy to manipulate as *Escherichia coli* and *S. cerevisiae*. The tightly regulated alcohol oxidase I promoter and the robust respiratory growth of this system that facilitates high cell densities (4) contributes to the rapid acceptance of this system over other expression systems. Research using this system may facilitate the discovery of more viable therapeutic agents for cancer treatment from natural products with bioavailable, safe, cost-effective and minimal side-effect properties in the future. Thus, the present study aimed to employ this system for the establishment of multi-copy gene constructs of hTopI using the pPIC3.5K vector in *Pichia*.

Materials and methods

Production of a multi-copy number insert in Pichia by an in vivo strategy. Pichia, which had a multi-copy number of hTopI (target gene; insert), was first produced via an *in vivo* strategy. Each copy of hTopI was carried by the pPIC3.5K

Correspondence to: Dr Boon Yin Khoo, Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia (USM), H53, Jalan Inovasi, 11800 Penang, Malaysia
E-mail: boonyin@usm.my

Abbreviations: hTopI, DNA topoisomerase I; YPD, yeast extract peptone dextrose; BMGY, buffered glycerol complex medium; OD, optical density; BMMY, buffered methanol-complex medium; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid

Key words: gene copy number, protein expression, enzyme activity, DNA topoisomerase I, *Pichia pastoris*

plasmid (9.0 kb; Invitrogen; Thermo Fisher Scientific, Inc.). In the process, the complete nucleotide sequence encoded hTopI that had been generated in a previous study (5) was excised from a pPICZα-A-hTopI plasmid (Invitrogen; Thermo Fisher Scientific, Inc.) with the restriction endonucleases *Eco*RI (Thermo Fisher Scientific, Inc.) and *Not*I (Thermo Fisher Scientific, Inc.) in two separate enzymatic reactions. The sequence was then inserted into the pPIC3.5K plasmid at the *Eco*RI and *Not*I sites. The construction of the recombinant plasmid was then viewed by agarose gel electrophoresis and confirmed by sequencing (First BASE Laboratories Sdn. Bhd.). The extracted recombinant pPIC3.5K-hTopI plasmid was then linearized by digestion with restriction endonuclease *Sal*I (Thermo Fisher Scientific, Inc.) prior to transformation into *Pichia* strain GS115 supplied by the Multi-Copy *Pichia* Expression kit (cat. no. K1750-01; Invitrogen; Thermo Fisher Scientific, Inc.). The yeast transformants were then screened on agar plates containing various concentrations of Geneticin® (Invitrogen; Thermo Fisher Scientific, Inc.). The transformants that survive at a higher concentration of Geneticin® are said to have more copies of recombinant plasmids transferred into yeast cells. The yeast colonies were subjected to copy number insert screening as described below.

Screening the multi-copy number insert Pichia transformants. The copies of recombinant plasmid in the yeast transformants can be quantified using quantitative PCR (qPCR) (3) or based on the ability of the clone to survive on agar plates containing various Geneticin® concentrations. As the yeast transformants were first screened on agar plates containing various concentrations of Geneticin®, agar plates containing various concentrations of Geneticin® were used to avoid any mismatch to screen the copy number insert of the transformants. A 200 μl aliquot of sterile yeast extract peptone dextrose (YPD; Merck KGaA) broth was added to each microtiter plate well using the aseptic technique. Each well of the first microtiter plate was inoculated with a single colony of the selected transformant (from the above section) using sterile toothpicks to resuspend the cells. The cell suspension in the microtiter plate was incubated at 30˚C without shaking. After 2 days, 10 μl of each culture were added to 190 μl sterile YPD broth in a new microtiter plate with the orientation marked as the first microtiter plate to keep track of the wells. The microtiter plate was incubated again as above. The process was repeated using the third microtiter plate to adjust the culture to be approximately similar visually to ensure equivalent numbers of cells were spotted on Geneticin® contained YPD plates. Following overnight incubation, 10 μl cell suspension from each well of the third microtiter plate was spotted on YPD plates containing 0 (control), 0.25, 0.50, 0.75 and 1.00 mg/ml Geneticin®. The cells were spotted on the agar in a regular pattern with a grid underneath the plate to ensure that an equal intensity of cells was spotted on the grid. The cell suspension liquid was allowed to soak in the gel, and the plates were then incubated at 30˚C for 2-5 days to allow the colonies of Geneticin-resistant transformants to grow on the respective plates in the grid. Single colonies that could survive at different concentrations of Geneticin® were selected, followed by re-streaking the selected colonies on respective YPD plates containing Geneticin®.

Growth induction of the multi-copy number insert Pichia transformants. A single antibiotic-resistant colony of *Pichia* transformants (GS115-pPIC3.5K-hTopI) from each YPD plate containing Geneticin® (as described above) was inoculated in 5 ml YPD medium. The cells were grown at 30˚C overnight with shaking at 200 rpm. In a 1-liter flask, 250 ml fresh buffered glycerol complex medium (BMGY; Merck KGaA) was inoculated with 250 μl of the overnight culture. The culture was grown again overnight until the optical density (OD) at 600 nm reached 2.0. The culture was harvested by centrifugation at 3,000 x g for 5 min at room temperature. The sample supernatant was then removed, and the cell pellet was resuspended in 10 ml buffered methanol-complex medium (BMMY; Merck KGaA). The cell suspension was diluted with BMMY to a final volume of 25 ml or until the culture reading at 600 nm reached 1.0. The cells were induced with 0.5% methanol every 24 h at 30˚C for 96 h. The cells were collected every 12 h to measure the cell density using a Multiskan Spectrum Spectrophotometric Plate Reader (Thermo Electron Co.) that represented growth. The OD_{600} was adjusted to 2.0 at 0 h of cultivation. The cells were also collected every 12 h on ice for total protein and specific protein sample preparation.

Analysis of specific protein expression in the Pichia transformants by western blot analysis. For the cell pellets collected from each ml of sample on ice (as described above), 100 μl breaking buffer and an equal volume of acid-washed glass beads were added. The mixture was vortexed for 30 sec and then incubated for a further 30 sec at room temperature for a total of 10 cycles. The mixture was then centrifuged at maximum speed (25x100 g) for 10 min at 4˚C. The clear supernatant was transferred to a fresh microcentrifuge tube for protein concentration analysis using the Pierce™ BCA Protein Assay kit (Bio-Rad Laboratories, Inc.) or stored the supernatant at -80˚C until use. The total protein concentration (mg/ml) for the sample collected every 12 h was measured. As for the specific protein, a total of 20 μg protein from each selected sample was then used for analysis by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the distributed proteins on the gel were transferred onto a nitrocellulose membrane (GE Healthcare; Cytiva) by the semidry transfer method using a Trans-blot SD Semidry Transfer Cell (Bio-Rad Laboratories, Inc.). Following protein transfer, non-specific proteins on the membrane were blocked with 5% blocking solution for 2 h on a shaker. The membrane was then washed with TBST three times for 10 min each. The membrane was incubated with purified mouse anti-hTopI antibody (1:5,000 dilution in TBST; cat. no. 556597; BD Biosciences) at 4˚C overnight. The following morning, the membrane was washed again with TBST as described above. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000 dilution in TBST; cat. no. 554002; BD Biosciences) for 1 h on a shaker. The membrane was then washed again with TBST as described above. The protein signal development process was carried out in a dark room, whereby the membrane was first overlaid with chemiluminescence substrate (Thermo Fisher Scientific, Inc.). Cling wrap was then overlaid on the wet membrane, followed by X-ray film (GE Healthcare; Cytiva) exposure for ~5 min. The film was then immersed in developer solution (MilliporeSigma) for a few

seconds, rinsed with tap water and then fixed with fixer solution (MilliporeSigma) for a few seconds. The developed film was then air-dried, and the signals on the X-ray film were scanned using ImageScanner III LabScan 6.0 (GE Healthcare; Cytiva).

Determination of recombinant enzyme activity by DNA relaxation assay. The protein samples, as prepared above, were also used for enzyme activity determination by DNA relaxation assay. The assay was performed in a total volume of 20 μl of reaction mixture containing 4 μl 5X hTopI reaction buffer, 1 μl 0.25 μg pBR322 plasmid DNA (Invitrogen; Thermo Fisher Scientific, Inc.) and 1 μl of the protein sample (6). Water was used instead of the protein sample in the reaction mixture for the negative control. All reactions were incubated at 37˚C for 30 min. Subsequently 4 μl 6X hTopI stopping buffer [3% SDS (Bio-Basic Inc.), 60 mM ethylenediaminetetraacetic acid (EDTA; Amresco), 50% glycerol (Bio-Basic Inc.) and 0.25% bromophenol blue] was added to the reaction mixture to stop the reactions. The reaction mixture was then briefly centrifuged and electrophoresed on a 1% (w/v) agarose gel in 0.5X TBE at 30 V for 200 min. The gel was then stained with ethidium bromide for 30 min and de-stained by soaking it in distilled water for a further 30 min. The gel was viewed under ultraviolet (UV) light and photographed using an Alpha Innotech Fluorchem FC2 (Thermo Fisher Scientific, Inc.). The enzyme action is expressed as B/A x 10^6 UI^{-1}, whereby A is an appropriate volume of supernatant added to the reaction. By contrast, B is the dilution factor of the supernatant required to complete the relaxation of 0.25 μg of pBR322 plasmid DNA.

Statistical analysis. The data are presented as the mean ± SD of triplicate determinations. The student's t-test was used to analyse the enzyme activity to compare mean values between two datasets. By contrast, GraphPad Prism version 9.4.0 one-way ANOVA with Tukey's multiple comparisons test (GraphPad Software, Inc.) was used to compare differences in the mean among groups of cell density and total protein concentration. The level of significance was set at α=0.05 (95% confidence interval), a value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

The constructed recombinant plasmid for in vivo strategy yeast transformation. A single copy of the hTopI fragment (~2,717 bp) that was ligated into the pPIC3.5K plasmid (~8,575 bp) at the *Eco*RI and *Not*I sites, producing the pPIC3.5K plasmid containing a single copy of hTopI (pPIC3.5K-hTopI) is illustrated in Fig. 1. The recombinant pPIC3.5K-hTopI plasmid, which was expected to be ~11,292 bp in length, was then linearized with the *Sal*I prior to the transformation of *Pichia* via an *in vivo* strategy to determine the effect of gene copy number on cell growth and total protein expression in the yeast transformants. The pPIC3.5K-hTopI plasmid was digested with *Eco*RI and *Not*I to confirm that all extracted plasmids harbored the correct insert. The extracted plasmid DNAs were also sent for sequencing to verify that each correct insert of the plasmid was cloned. The nucleotide sequence (GenBank: MW117125.1; https://www.ncbi.nlm.nih.gov/nuccore/MW117125) of the insert in the plasmid is illustrated in Fig. 2. The results indicated that the recombinant pPIC3.5K-hTopI plasmid was constructed.

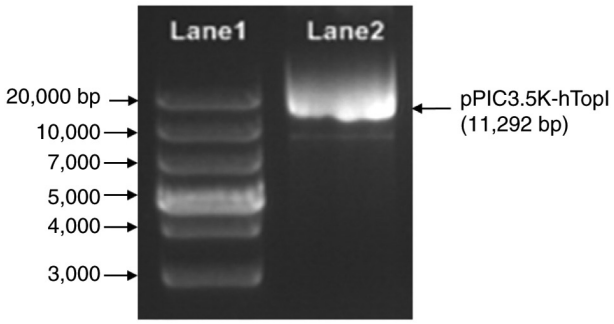

Figure 1. Construction of recombinant plasmid via *in vivo* strategy. A 0.7% agarose gel electrophoresis of the constructed recombinant pPIC3.5K-hTopI plasmid DNA was performed. Lane 1, GeneRuler 1 kb Plus DNA ladder; lane 2, recombinant pPIC3.5K-hTopI plasmid. Of note, a cropped image of the gel is presented; the full-length gel can be presented upon reasonable request.

In vivo screening of multi-copy insert transformants. The multi-copy inserts of His^+ transformants were produced after the linearized pPIC3.5K-hTopI plasmid DNA was transformed into the *Pichia* GS115 strain. As many as 68 yeast transformants (GS115-pPIC3.5K-hTopI) were selected for subculturing from the microtiter plate. From the observations, clones 46, 55, 19 and 50 were selected from the plates that contained 0.25, 0.50, 0.75 and 1.00 mg/ml Geneticin, respectively (Fig. 3). The selection was based on the ability of the clones to survive on agar plates containing various antibiotic concentrations. The *Pichia* transformants (GS115-pPIC3.5K-hTopI), which were constructed via an *in vivo* strategy and resistant to various Geneticin concentrations, were later subjected to cell growth (density) and total protein expression analyses.

Cell growth and total protein expression of the transformants. The cell density of the collected cultures was measured every 12 h, whereby the cell density increased with the incubation times (Fig. 4A). The highest cell density was observed in GS115-pPIC3.5K-hTopI resistant to 0.50 mg/ml Geneticin at 96 h of incubation. The absorbance of the cell density of the His^+ transformants at this time point was 11.39 units ($P<0.001$) at OD_{600}. However, increasing the number of inserts and incubation time did not increase the cell density of the transformants. The transformants resistant to 0.25 mg/ml Geneticin at 60 and 84 h of incubation exhibited similar cell densities: 11.19 units ($P<0.01$) and 11.33 units ($P<0.01$) at OD_{600}, respectively. Conversely, the increase in insert copy number and incubation time did increase the expression level of total protein in the transformants. In brief, the highest total protein expression was obtained in the clone that was resistant to 1.00 mg/ml Geneticin at 48 h of incubation and 0.75 mg/ml Geneticin at 60 h of incubation (Fig. 4B). The total protein expression levels in these transformants were 1.76 and 1.75 mg/ml ($P<0.001$), respectively. Other GS115-pPIC3.5K-hTopI strains that exhibited similar total protein expression levels were also observed in the transformants resistant to 1.00 mg/ml Geneticin at 24 h of incubation (1.70 mg/ml) and 1.00 mg/ml Geneticin at 84 h of incubation (1.72 mg/ml). In summary, the level of total protein

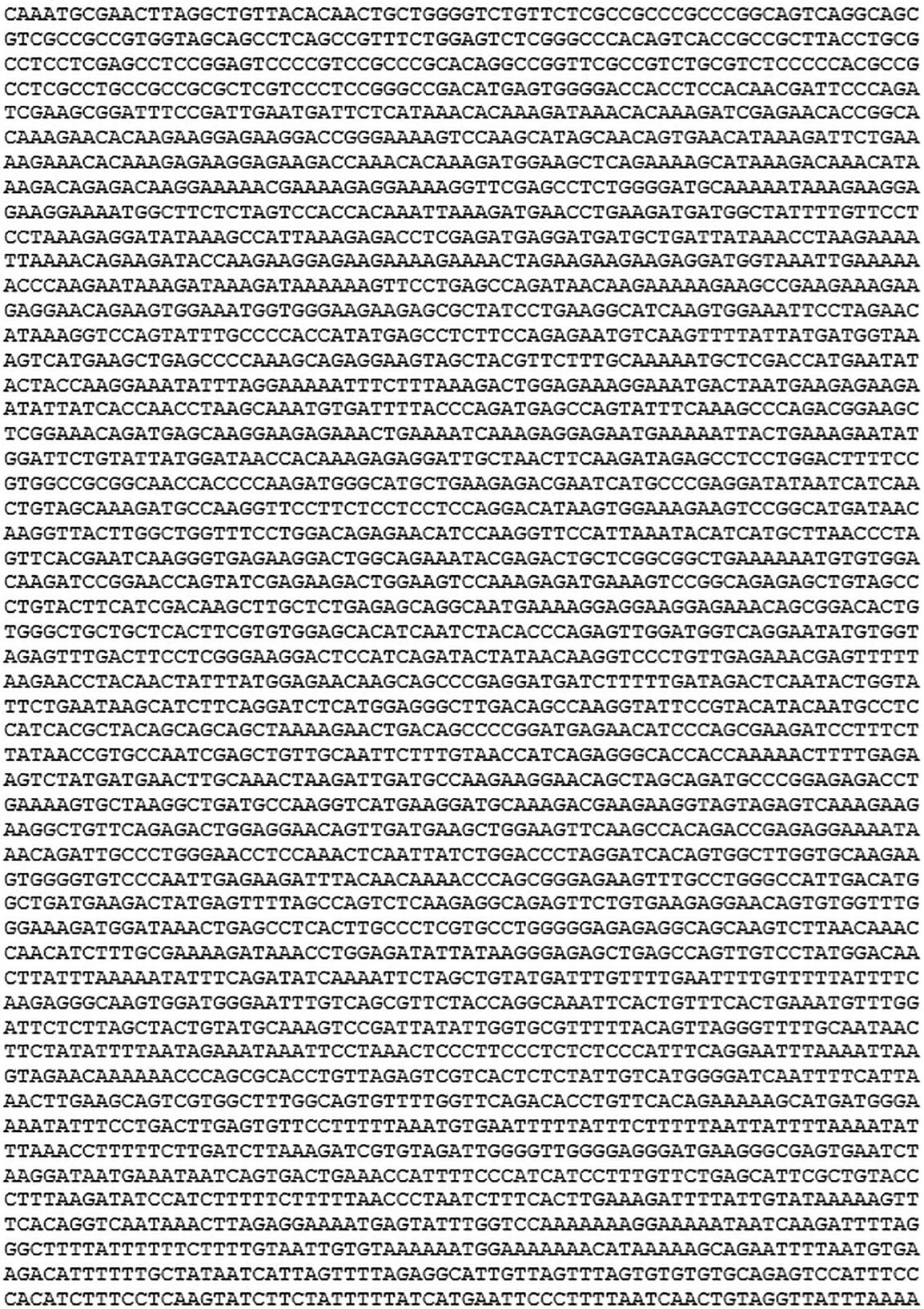

Figure 2. Nucleotide sequence of hTopI in the pPIC3.5K-hTopI plasmid. The sequence was identified by sequencing and has been deposited in GenBank (GenBank: MW117125.1). The sequence can be accessed at https://www.ncbi.nlm.nih.gov/nuccore/MW117125.

expression in the clones of GS115-pPIC3.5K-hTopI, which were resistant to various concentrations of Geneticin, indicated that GS115-pPIC3.5K-hTopI exhibited the highest level of total protein expression and resistance to 0.25 mg/ml Geneticin was the clone collected at 24 h of incubation (1.60 mg/ml). The GS115-pPIC3.5K-hTopI that exhibited the highest level of total protein expression and resistance to 0.50 mg/ml Geneticin was the clone collected at 36 h of incubation (1.68 mg/ml), the

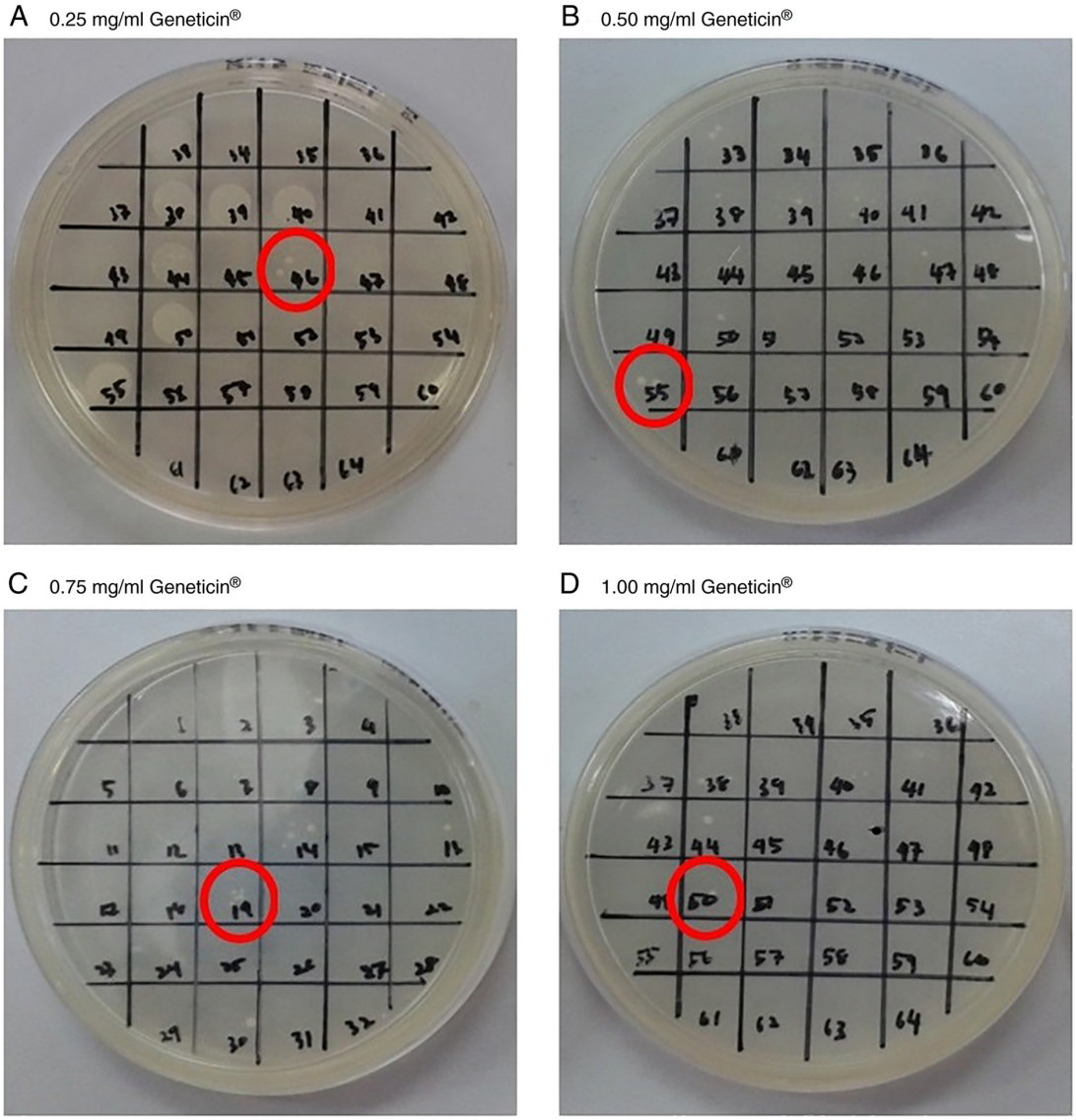

Figure 3. Screening of GS115-pPIC3.5K-hTopI on YPD agar containing various Geneticin concentrations. The concentrations of antibiotic used on the YPD agar were (A) 0.25, (B) 0.50, (C) 0.75 and (D) 1.00 mg/ml. The grid circled in red indicates the clone resistant to the antibiotic's particular concentration in the agar plate. Images of agar gel were derived from the same experiment and were processed in parallel.

GS115-pPIC3.5K-hTopI that exhibited the highest level of total protein expression and resistance to 0.75 mg/ml Geneticin was the clone collected at 60 h of incubation (1.75 mg/ml), and the GS115-pPIC3.5K-hTopI that exhibited the highest level of total protein expression and resistance to 1.00 mg/ml Geneticin was the clone collected at 48 h of incubation (1.76 mg/ml) (Table I). These four clones were selected for comparisons in subsequent experiments. Although the highest expression level of total protein was found in the selected transformants, the normalization of the increased total protein expression level in all transformants with cell density per se did not reveal any statistically significant differences.

Specific protein expression and enzyme activity of hTopI on selected clones. The expression analysis revealed that a protein band of ~91 kDa was observed in the total protein of all four selected clones (Fig. 5A). The 91 kDa protein was detected by the antibody purified from mouse anti-human DNA TopI, indicating that the protein of interest was expressed in the multi-copy gene of His^+ transformants using the *in vivo* strategy (Fig. 5B). The clones of GS115-pPIC3.5K-hTopI resistant to 0.25 mg/ml Geneticin and were collected at 24 h of incubation, those resistant to 0.50 mg/ml Geneticin and were collected at 36 h of incubation, those resistant to 0.75 mg/ml Geneticin and were collected at 60 h of incubation, and those resistant to 1.00 mg/ml Geneticin and were collected at 48 h of incubation; these were then subjected to the determination of the activity of hTopI. The hTopI activity of the clones was assayed based on the ability of hTopI to relax pBR322 supercoiled DNA. It was found that hTopI expressed by GS115-pPIC3.5K-hTopI resistant to various Geneticin concentrations could relax the pBR322 supercoiled DNA compared with the supercoiled DNA in the reaction without hTopI (Fig. 5C). The supercoiled DNA migrated more rapidly than the relaxed form of DNA due to the smaller size of the DNA, and hence, the band of supercoiled DNA appeared lower than the relaxed form of the DNA on the same agarose gel. The enzyme activity of hTopI in GS115-pPIC3.5K-hTopI resistant to various Geneticin concentrations is also summarized in Table II. The highest

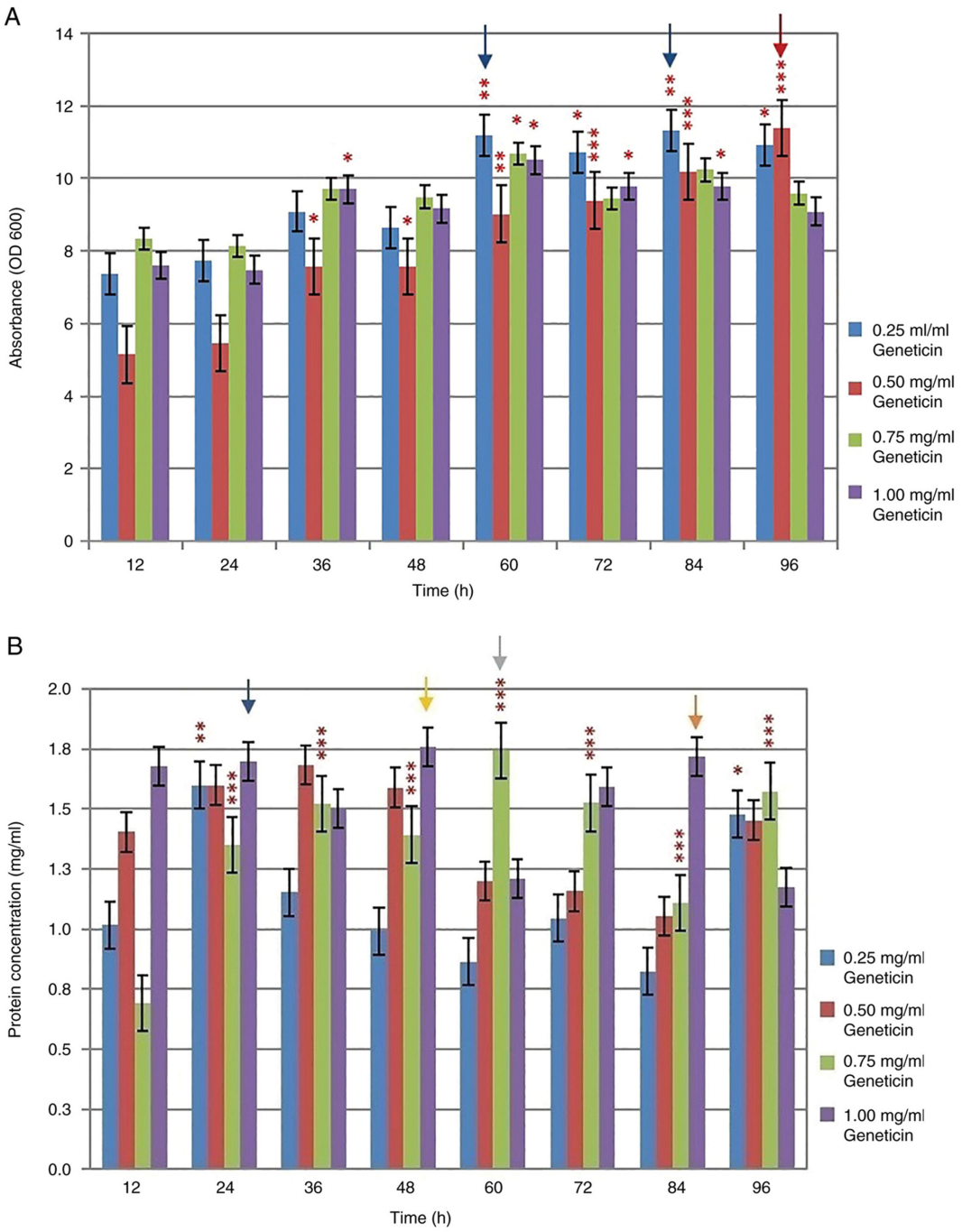

Figure 4. Cell growth and total protein expression of GS115-pPIC3.5K-hTopI. (A) Cell growth of the transformants following 96 h of incubation. The density of GS115-pPIC3.5K-hTopI resistant to various Geneticin concentrations on agar plates was measured using a Multiskan Spectrum Spectrophotometric Plate Reader. (B) Total protein expression of the transformants at 96 h of incubation. The Pierce™ BCA protein assay was used to measure the total protein expression level in GS115-pPIC3.5K-hTop1 resistant to various Geneticin concentrations. All data represent the mean ± SD of three replications. *P<0.05, **P<0.01 and ***P<0.001 vs. the 12-h group.

enzyme activity of hTopI was observed in the culture expressed by GS115-pPIC3.5K-hTopI resistant to 1.00 mg/ml Geneticin ($19.7x10^4$ Ul/OD_{600}). The enzyme activity was ~3-fold greater than the enzyme activity of hTopI in GS115-pPIC3.5K-hTopI resistant to 0.25 mg/ml Geneticin ($7.74x10^4$ Ul/OD_{600}). The enzyme activities of hTopI in GS115-pPIC3.5K-hTopI resistant to 0.50 and 0.75 mg/ml Geneticin were $10.6x10^4$ Ul/OD_{600} and $9.35x10^4$ Ul/OD_{600}, respectively (Table II). This phenomenon demonstrated that the enzyme activity of hTop1 produced in GS115-pPIC3.5K-hTopI was likely to be proportional to the level of antibiotic resistance or increased with the target gene's increment copy number in each clone.

Discussion

In the present study, the recombinant pPIC3.5K-hTopI plasmid, which contained a copy of the human DNA topoisomerase I (hTopI), was constructed. The *Pichia* transformants or recombinant yeast (GS115-pPIC3.5K-hTopI), which contained a multi-copy number of hTopI, was also produced via an *in vivo*

Table I. Cell density and total protein expression by GS115-pPIC3.5K-hTopI resistant to various Geneticin concentrations.

Time point (h)	Geneticin® concentration (mg/ml)	Cell density (absorbance OD_{600})	Total protein (mg/ml)	Total protein per hour (mg/ml/h)	Total protein per cell density (mg/ml/unit)
12	0.25 mg/ml	7.38	1.02	0.08	0.14
	0.50 mg/ml	5.14	1.40	0.12	0.27
	0.75 mg/ml	8.33	0.69	0.06	0.08
	1.00 mg/ml	7.60	1.68	0.14	0.22
24	0.25 mg/ml	7.75	1.60[a]	0.07	0.21
	0.50 mg/ml	5.45	1.60	0.07	0.29
	0.75 mg/ml	8.15	1.35	0.06	0.17
	1.00 mg/ml	7.48	1.70[b]	0.07	0.23
36	0.25 mg/ml	9.09	1.15	0.03	0.13
	0.50 mg/ml	7.57	1.68[a]	0.05	0.22
	0.75 mg/ml	9.72	1.52	0.04	0.16
	1.00 mg/ml	9.70	1.50	0.04	0.15
48	0.25 mg/ml	8.65	0.99	0.02	0.11
	0.50 mg/ml	7.56	1.59	0.03	0.21
	0.75 mg/ml	9.49	1.39	0.03	0.15
	1.00 mg/ml	9.16	1.76[a,b]	0.04	0.19
60	0.25 mg/ml	11.19[b]	0.86	0.01	0.08
	0.50 mg/ml	9.02	1.20	0.02	0.13
	0.75 mg/ml	10.69	1.75[a,b]	0.03	0.16
	1.00 mg/ml	10.50	1.21	0.02	0.12
72	0.25 mg/ml	10.72	1.04	0.01	0.10
	0.50 mg/ml	9.39	1.16	0.02	0.12
	0.75 mg/ml	9.44	1.53	0.02	0.16
	1.00 mg/ml	9.78	1.59	0.02	0.16
84	0.25 mg/ml	11.33[b]	0.82	0.01	0.07
	0.50 mg/ml	10.18	1.05	0.01	0.10
	0.75 mg/ml	10.23	1.11	0.01	0.11
	1.00 mg/ml	9.78	1.72[b]	0.02	0.18
96	0.25 mg/ml	10.93	1.48	0.02	0.14
	0.50 mg/ml	11.39[b]	1.45	0.02	0.13
	0.75 mg/ml	9.59	1.57	0.02	0.16
	1.00 mg/ml	9.08	1.18	0.01	0.13

Cell density and total protein concentrations were measured by spectrophotometry and Bio-Rad protein assays. All clones were cultured on antibiotic contained agar plates for 96 h. [a]Clones that were selected for subsequent experiments; [b]high level of cell density and total protein expression in the cultures. The normalization of the increased total protein expression level per hour and the increased total protein expression level per cell density in all transformants did not reveal any statistically significant differences ($P>0.05$).

strategy. The cell density of GS115-pPIC3.5K-hTopI was likely to be unaffected by the copy number of hTopI. However, the total protein expression and the target enzyme activity of the recombinant yeast were increased in accordance with the increased copy number of hTopI in the host, whereby the yeast that was able to survive at the highest concentration of Geneticin expressed the highest level of total protein and had the highest activity of the enzyme.

Pichia is a widely used host system for the expression of heterologous proteins. In addition to the popularity factors, this system also offers the strong and highly regulated alcohol oxidase promoter, stable integration events in the host chromosomal DNA and efficient techniques for high-density cultivation to express the protein of interest. Therefore, the present study utilized this yeast system to express hTopI, whereby the gene encoding the protein of interest is ~2,298 bp. The expression using recombinant yeast containing a single copy number of the target gene was disappointingly low; indeed, the multi-copy number of the gene expression cassette has been one of the most effective strategies to increase the expression of the GOI (3,7). The recombinant yeast was constructed using the pPIC3.5K vector in the present study

Table II. The activity of hTopI in the clones of GS115-pPIC3.5K-hTopI resistant to various Geneticin concentrations.

Clone resistant to Geneticin® (mg/ml)	OD_{600} of culture	Intracellular enzyme activity (Ul/OD_{600})	Total enzyme activity (U/l)
0.25	7.75	$7.74x10^4$	$3.02x10^6$
0.50	7.57	$10.6x10^4$	$4.02x10^6$
0.75	10.69	$9.35x10^4$	$5.02x10^6$
1.00	9.16	$19.7x10^4$	$9.04x10^{6a}$

The enzyme activity is expressed as the volume of supernatant induced in the reaction over the dilution factor of the supernatant required to complete the relaxation assay $x10^6$ U/l. [a]P<0.05, indicating a statistically significant difference relative to the value in the clone resistant to 0.25 mg/ml Geneticin®.

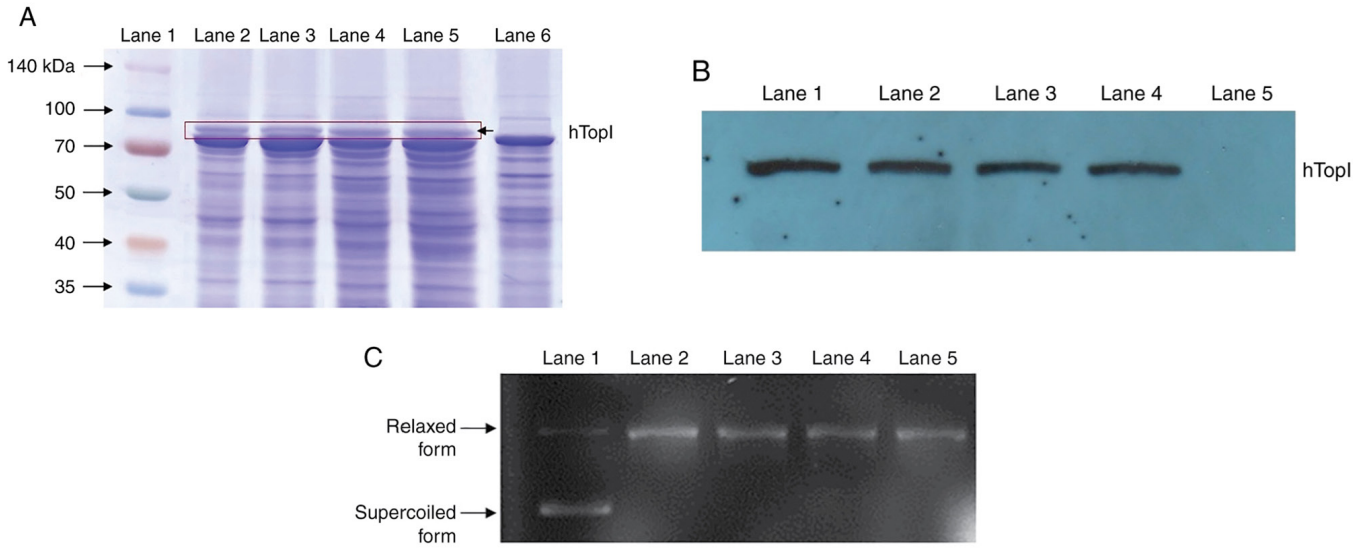

Figure 5. Specific protein expression for selected clones. (A) SDS-PAGE of total protein extracted from GS115-pPIC3.5K-hTopI resistant to various Geneticin concentrations and collected at different incubation time points. Lane 1, Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific, Inc.); lane 2, clone resistant to 1.00 mg/ml Geneticin, collected at 24 h of incubation; lane 3, clone resistant to 1.00 mg/ml Geneticin, collected at 48 h of incubation; lane 4, clone resistant to 0.75 mg/ml Geneticin, collected at 60 h of incubation; lane 5, clone resistant to 1.00 mg/ml Geneticin, collected at 84 h of incubation; lane 6, negative control. The marker was run on the same gels/blots. (B) Western blot analysis for hTopI protein detection in GS115-pPIC3.5K-hTopI resistant to various Geneticin concentrations and collected at different incubation time points. Lane 1, clone resistant to 1.00 mg/ml Geneticin, collected at 24 h of incubation; lane 2, clone resistant to 1.00 mg/ml Geneticin, collected at 48 h of incubation; lane 3, clone resistant to 0.75 mg/ml Geneticin, collected at 60 h of incubation; lane 4, clone resistant to 1.00 mg/ml Geneticin, collected at 84 h of incubation; lane 5, negative control. (C) A 1.0% agarose gel electrophoresis of the enzyme activity of hTopI in the clones that were resistant to various Geneticin concentrations and collected at different incubation time points. Lane 1, supercoiled DNA of pBR322 incubated without hTopI (control); lane 2, supercoiled DNA of pBR322 incubated with hTopI from the clone resistant to 0.25 mg/ml Geneticin (24 h); lane 3, supercoiled DNA of pBR322 incubated with hTopI from the clone resistant to 0.50 mg/ml Geneticin (36 h); lane 4, supercoiled DNA of pBR322 incubated with hTopI from the clone resistant to 0.75 mg/ml Geneticin (60 h); lane 5, supercoiled DNA of pBR322 incubated with hTopI from the clone resistant to 1.00 mg/ml Geneticin (48 h). The control was run on the same gels/blots.

via the *in vivo* strategy to determine the effects of gene copy number on cell density, the expression of total protein and the target enzyme activity in *Pichia*.

His^+ *Pichia* transformants with multi-copy inserts (recombinant yeast) resistant to various concentrations of Geneticin were also selected in the present study. The gene copy number can be quantified using qPCR (3) or it can be estimated based on the ability of the clones to survive on agar plates containing various antibiotic concentrations. As described in the study by Athmaram *et al*, they selected a panel of *Pichia* clones carrying increasing copies of the heterologous gene based on Geneticin resistance and the SYBR-Green-based qPCR approach was more or less matched (8). Overall, the recombinant *Pichia* transformants carrying a maximum of four to six copies of the transgene were identified using these strategies. The association between the Geneticin resistance level and PCR positivity for screening the *Pichia* transformants revealed that the clones capable of surviving on agar plates containing 0.25, 0.50 and 0.75 mg/ml Geneticin may contain 1, 2 and 4-6 gene copy numbers, respectively. From that, it can be estimated that the clones capable of surviving on agar plates containing 1.00 mg/ml Geneticin may contain a gene copy number >4-6. However, the cell density of the selected clones was likely not affected by the copy number of the target gene in the host, which may be not be affected by the downstream metabolic activity of the cells. According to previous studies,

gene expression induction results in excessive plasmid replication that consequently increases the plasmid copy number in the transformants (9,10). However, this phenomenon also contributes to the host cell metabolic burden (11). As a result, the metabolic activity is strongly impaired in the cells, indicated by the decelerated increase in biomass and OD. For the effect of the *in vivo* strategy, the present study found the highest expression level of total protein (as much as 1.76 mg/ml) in GS115-pPIC3.5K-hTopI resistant to 1.00 mg/ml Geneticin at 48 h of incubation. However, normalization of the total protein level per hour and per cell density in each transformant was statistically insignificant compared to the total protein level in control. Therefore, the study is continued by investigating the target enzyme activity, whereby the study found an increment in the gene copy number to increase the enzyme activity of hTopI produced in GS115-pPIC3.5K-hTopI.

A transformant or clone with two identical copies of a gene under the control of an identical promoter, in theory, should produce twice as much protein. However, increasing the gene dosage does not necessarily increase protein expression. In some cases, e.g., human trypsinogen (12) and Na-ASPI (13), increased the gene dosage reduced the protein expression. Therefore, an optimal level rather than a maximal copy number should be considered due to other possible protein expression bottlenecks, e.g., protein translation, secretion or degradation (12,14,15). Furthermore, an increased copy number of foreign genes may result in the alteration of normal metabolism in *Pichia*, leading to a negative influence on the normal cell physiology of multiple-copy recombinant yeast, particularly in the case of secretory expression, which includes a reduction in methanol consumption capacity and specific growth rate, decreased cell viability, increased instability of integrated foreign genes or diminished cell secretory ability (16). For this reason, it is suggested to test the transformants with increasing gene copy numbers and later identify the optimal gene copy number for maximum protein production. Although the strategy used in the present study did not significantly alter the expression of total protein per cell density in each clone, the ability of hTopI expressed by GS115-pPIC3.5K-hTopI resistant to various Geneticin concentrations was increased as the resistance towards Geneticin was increased. This event also further demonstrated that the hTopI expression ability in the present study was able to relax supercoiled DNA, and the enzyme activity increased with increasing target gene copy number. It is hypothesized that after several rounds of subcultures, the genes in multiple copies may not be retained and therefore need to be removed. Indeed, the gene copy number can be ascertained by maintaining the clones on the agar plates containing respective concentrations of Geneticin.

In conclusion, the present study provides information on producing target protein from the recombinant *Pichia* using only a shaker flask system, which can be further developed as an in-house resource for screening potential anticancer agents from various natural resources.

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Availability of data and materials

The datasets used and/or analysed during the current study, including the nucleotide sequence of hTopI in the pPIC3.5K-hTopI plasmid that had been deposited in GenBank (GenBank: MW117125.1; https://www.ncbi.nlm.nih.gov/nuccore/MW117125), are available from the corresponding author upon reasonable request.

Authors' contributions

KBY contributed to the conception and design of the study. NAF and LSK performed the experiments under technical support provided by CAL and KBY. In addition, KBY interpreted the results, and drafted and revised the manuscript. CAL and KBY confirm the authenticity of all the raw data. All authors have read and agreed to the final version of this manuscript submitted for publication.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declares that they have no competing interests.

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