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Full Length Research Paper

Long-term stability of β-galactosidase protein expression in the absence of selective growth conditions in transfected Chinese hamster ovary cell

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In this study, the adequacy of β-galactosidase (β-gal) as marker for models that requires durable and high level gene expression in the absence of selective pressure was investigated. Chinese hamster ovary (CHO) cells were transfected with expression vector pcDNA4/HisMax-TOPO/*lac*Z containing *lac*Z and zeocin resistant genes. 26 recombinant CHO cell lines were established using lipid cationic transfection (TransFast™ Transfection Reagent) as DNA transfer method. 6 clones were productive in the expression of β-gal when grown in the presence of zeocin as the selective pressure. 2 sub-clones, TF8 (1) and TF9 (7) grown for 11 passages in nonselective medium which maintained high levels of protein expression with specific β-gal activity in the absence of zeocin were almost constant at 1.5704 and 4.3017 units β-gal activity/mg protein respectively. Specific growth rate of TF8 (1) and TF9 (7) in the absence of zeocin were approximately 0.638 and 0.656 day^{-1} respectively. The expression of β-gal does not affect the cell growth and the transfectants were stable population in terms of cell viability. Removal of zeocin from the media increases specific growth rate from a range of 24 to 52% and β-gal protein production in reference to specific activity increases from 128 to 320% with the capability to be expanded to larger volumes. In this study, we demonstrate transfected CHO cells with the ability to produce β-gal without the presence of zeocin for at least 11 passages.

Key words: Chinese hamster ovary (CHO), β-galactosidase, clone stability, selective pressure.

INTRODUCTION

β-Galactosidase (β-gal) is a convenient reporter molecule to monitor gene and transfection efficiency in broad spectrum of model organism (Naylor, 1999). β-gal is the product of the *lac*Z gene of *Escherichia coli* and this enzyme is composed of 1024 amino acids (Gong et al., 2009). β-gal monomers (116 kDa) are large proteins that tetramerized to form the active site of the enzyme. Applications using β-gal as a reporter molecule include transcription studies (Buteau et al., 2006); enhancer, promoter and gene trap applications (Watt et al., 2001) as well as fusion protein (Rönnmark et al., 2003). The natural reaction catalyzed by β-gal is to hydrolyze the disaccharide lactose to galactose and glucose at an optimal pH of 7.2.

The ability of β-gal to hydrolyze β-D-galactopyranoside analogs, such as o-nitrophenyl-β-D-galactopyranoside (ONPG), chlorophenol red β-D-galactopyranoside (CPRG) and 9H- (1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside (DDAOG) for cell-based assays (Buller et al., 2003; Kim et al., 2004; Gong et al., 2009)

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Abbreviations: β-gal, β-Galactosidase; **CHO,** Chinese hamster ovary.

and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) for histological analysis (Engelmann et al., 2006; Wang et al., 2009) allows reliable quantification of reporter enzyme expression over a wide range of applications using simple colorimetric assays. More sensitive β-gal assay methods based on the fluorogenic substrate fluorescein di-β-D-galactopyranoside (FDG), 4-methylumbelliferyl-β-D-galactopyranoside (MUG) (Zhang et al., 1991; Gary and Kindell, 2005) and the chemiluminometric substrate galacton (Martin et al., 1996) have also been developed.

β-Gal has the advantage over chloramphenicol acetyl-transferase (CAT) in that the assay tends to be a simpler colorimetric assay and does not involve the use of radioisotopes. Other types of reporter genes are less convenient, requiring either radioactive substrates (Celen et al., 2009) or special equipment to measure lumine-scence or fluorescence (Branchini et al., 2010; Zhang and Yang, 2011). The utility of this protein in experimental biology is being defined in a variety of cells. For example, β-gal was used to monitor transfection studies of *Toxoplasma gondii (Tg)* and as a readily screenable marker for stable transformation (Seeber and Boothroyd, 1996). β-Gal have been used to monitor transfection efficiency in hepatocyte and hepatoma cells (Sun et al., 2004). β-Gal has also been used to assess gene transfer techniques in ocular cell of human eyes (Jian et al., 2000).

The stability of cell line used is a critical parameter in large scale production of recombinant protein. To ensure predictable high recombinant protein expression during production, the stability of this cell line in the absence of selection pressure is an important criterion (Wurm, 2004). When drug resistant cells are propagated in the absence of the selective agent, the amplified genes may be maintained or lost. Changes in recombinant CHO cell production after extended culture in the presence or absence of selective pressure have been reported (Chusainow et al., 2009; Kim et al., 1998; Morrison et al., 1997). Numerous publications (Subramanian et al., 2001; Poluri et al., 2005) have proven the usefulness of β-gal as reporter molecule in the setting of transient and stable gene expression. The levels of β-gal activity assayed in clonal isolates can be used to identify clones producing high levels of the protein of interest (Grossman et al., 1997). However, it remains unclear whether cell lines are able to produce higher level of β-gal expression over many passages in the absence of selective growth conditions.

In this study, we generated several subclones of recombinant CHO expressing β-gal protein and analyzed the higher producer clones regarding their growth characteristics, protein productivity and their stability in long term culture. The aim of this study was to generate stable recombinant CHO cells which maintain high expression levels of β-gal for longer duration period after the removal of selectable marker. It will also enable us to determine the effect of β-gal on cell growth the absence of the additional selectable marker.

MATERIALS AND METHODS

Plasmid DNA preparation

Plasmid pcDNA4/HisMax-TOPO/*lac*Z (8.35 kb) from Invitrogen (USA) is a eukaryotic expression vector containing the CMV promoter and *lac*Z gene that codes for the enzyme β-gal that allows quick determination of cells expressing the gene. The plasmid was first transformed in *Escherichia coli* Top10 and prepared using the Pure Yield plasmid midiprep system (Promega, USA). The struc-tural integrity and topology of purified DNA plasmid were analysed by 0.8% agarose gel electrophoresis. DNA concentration and purity were quantified by UV absorbance at 260 and 280 nm on a Bio-photometer (Eppendorf, Germany).

Cell transfection

CHO cells were cultured in growth medium containing culture medium RPMI-1640 with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all purchased from Gibco-BRL). The cells were seeded at 1.6×10^5 cells per well onto 12 well plates 24 h before transfection. The cells were grown to 70% confluence at the time of transfection. The transfection condition used was based on a study by Saifudin et al. (2011) using 9 µl TransFast™ Trans-fection reagent and 1.5 µg of plasmid at a 2:1 charge ratio of lipid: DNA. The TransFast/DNA solutions were diluted in culture medium RPMI-1640 with no serum and the mixture was incubated for 15 min at room temperature. The culture medium was removed from the 12-well plates containing cells, washed twice by PBS and replaced by the TransFast/DNA mixture. After 1 h, 1.6 ml growth medium was added and the cells were incubated for 48 h. Stable transfected cells were selected by zeocin (Invitrogen, USA) at 300 g/ml in the growth medium for 3 weeks.

Cell cloning by limiting dilution

Transfected colonies were identified morphologically and marked before transferred to 96 well plates. Each well was supplied with 150 µl medium. The cells were expanded into 12 well plates when 100% confluence was reached.

Stability of protein expression in selective and non selective medium

In order to select the best producing clones for β-gal production and to monitor the improvement in β-gal productivity during the stepwise amplification process, different clones and cell pools were screened for their productivities using β-Red β-galactosidase assay kit. Two highest β-gal producer clones namely TF8(1) and TF9(7) were cultivated up to 11 passages started at the 21st passage. Both clones were seeded at 2.5×10^5 cells/ml in T25-flask and T75-flask containing selective media (RPMI 1640 + 5% FBS + 300 µg/ml zeocin) and non selective media (RPMI 1640 + 5% FBS) in two working volumes of 6 and 30 ml. Cell concentration of both clones and their β-gal activity were determined after each passage of every 4 days in culture.

β-Galactosidase activity assay

The assay was performed using BetaRed β-galactosidase assay kit

Table 1. β-Galactosidase activity (milliunits/mg protein) in zeocin resistant pools.

Clone	TF8(1)	TF8(3)	TF9(1)	TF9(2)	TF9(3)	TF9(7)
β-Gal activity (milliunits/mg protein)	36.1	8.63	4.08	7.49	5.82	52.45

(Novagen, USA). Cells were rinsed with PBS once and 200 µl of Reportasol extraction buffer was added to each well. The plate was shaken at room temperature for 5 min. For assay protocol, 5 to 50 µl cell extract and negative control were placed into different wells on the 96 well plates and 145 µl of BetaRed reaction buffer (with DTT and CPRG substrate) was added to the wells. The plate was incubated 37°C until the reaction changes to red. BetaRed stop buffer was added to stop the reaction and absorbance was measured at 595 nm.

Bradford protein assay

Total amount of protein was determined by Quickstart Bradford protein assay (Bio-Rad, USA) according to the manufacturer's protocol, using bovine serum albumin (BSA, Bio-Rad) as the protein standard. The protein standard (125 to 2000 mg/ml) and 20 µl cell lysates were incubated with Bradford dye reagent at room temperature for at least 5 min. The absorbance was measured at 595 nm using Bradford program on the Biophotometer (Eppendorf, Germany).

Western blot analysis

Cell extract was added with protein loading dye and boiled for 5 min at 100°C. A volume of 35 µl was loaded and separated by electrophoresis on 12% SDS-polyacrylamide gel. The gel was blotted onto a nitrocellulose membrane (Bio-Rad, USA). The membrane was blocked for 1 h using 5% skimmed milk in PBS with 0.05% Tween-20 and incubated overnight with anti-β-galactosidase monoclonal antibody (Promega, USA) diluted to 1:5000 in blocking buffer. After washing, the captured β-galactosidase protein was detected with horseradish peroxidase-conjugated anti-mouse IgG (H+L) (Promega, USA) diluted to 1:2500 in blocking buffer for 1 h at room temperature. The protein bands were detected using 3, 3',5,5'-tetramethylbenzidine solution (Promega, USA).

Measurement of cell concentration and viability

The cell concentration was measured using a Neubauer hemacytometer and the viable cells were detected using the trypan blue (Sigma) exclusion test. The cell viability was calculated by dividing the number of viable cells by the total number of cells.

Determination of specific growth rate and doubling time

The specific growth rate, μ, and doubling time were evaluated as described by Shuler and Kargi (1992) from data collected during the exponential growth phase and are defined as follows:

At exponential phase:

$$\frac{dX}{dt} = \mu.X, \qquad X = X_o \quad \text{at} \quad t = t_o \tag{1}$$

Where, $\frac{dX}{dt}$ is the population growth rate (cell ml^{-1} day^{-1}); μ is the specific growth rate (day^{-1}); X is the concentration of viable cells and t is the cultivation time.

At constant μ:

$$\ln X - \ln X_o = \mu.t \tag{2}$$

Cell doubling time (day) was based on Equation (3).

$$t_d = \frac{\ln 2}{\mu} \tag{3}$$

RESULTS

CHO cells were transfected using the TransFast reagent with pcDNA4/HisMax-TOPO/*lacZ*, followed by zeocin selection and single-cell cloning. All pools grew at equivalent rates for subsequent passages with doubling times of approximately 1.4 days and had passages twice weekly. A total of 26 single-cell clones were isolated by limiting dilution. 6 of those single-cell clones proliferated well and detected as producer clone. These clones were identified as TF8(1), TF8(3), TF9(1), TF9(2), TF9(3) and TF9(7). The transfected pools were able to survive due to the availability of at least one copy of a transcriptionally active *Sh ble* gene. β-gal activity varied considerably between transfections ranging from 4.08 to 52.45 milliunits/mg of the total zeocin resistant population (Table 1). Measurements of enzyme activity were made after the 1st passage in zeocin containing medium in 12 wells plate for each pool.

Two of the best producing clone, TF8(1) and TF9(7) were cultivated as monolayer cultures in the present and absence of zeocin. The cells were incubated for 4 days to ensure that cells are always within late exponential growth phase for maximum productivity. The cell growth and β-gal production of individual sub-clones were examined for 11 passages in 6 and 30 ml culture volumes. To assess the durability, zeocin was removed from the growth medium for subsequent 11 passages in culture. Table 2 summarizes the β-gal activity, viable cell concentration, specific growth rate and cell doubling time growth under non-selective conditions. In all after 11 passages of culture with zeocin and pools, a slight increase in the β-gal activity was noted under nonselective

Table 2. The β-gal activity, viable cell concentration, specific growth rate and cell doubling time of passage sub-clones at the 1st passage and the 31st passage during long term culture. Comparison was carried out in 6 ml and 30 ml working volumes of cultures.

Parameter	Culture V (ml)	TF8(1)			TF9(7)		
		Value on 1st passage[a]	Value on the 31st passage		Value on 1st passage[a]	Value on the 31st passage	
			Zeocin (+)	Zeocin (–)		Zeocin (+)	Zeocin (–)
β-gal activity (unit/mg)	6	0.2347 ± 0.19	0.1002 ± 0.06 (43%)	0.4011 ± 0.023 (171%)	0.3409 ± 0.24	0.3349 ± 0.070 (98%)	1.4284 ± 0.112 (419%)
	30	0.722 ± 0.29	0.3921 ±0.23 (54%)	1.5704 ± 0.077 (218%)	1.049 ± 0.08	1.0598 ± 0.12 (101%)	4.3017 ± 0.124 (410%)
Viable cell (×10^6 cell/ml)	6	1.63 ± 0.07	2.04 ± 0.29 (125%)	4.04 ± 0.30 (248%)	1.28 ± 0.09	1.49 ± 0.15 (118%)	4.12 ± 0.288 (322%)
	30	1.87 ± 0.05	1.93 ± 0.14 (103%)	3.21 ± 0.51 (172%)	1.97 ± 0.12	1.72 ± 0.36 (87%)	3.99 ± 0.35 (203%)
Specific growth rate, μ (day^{-1})	6	0.464 ± 0.09	0.532 ± 0.12 (114.7%)	0.645 ± 0.13 (139%)	0.508 ± 0.12	0.448 ± 0.16 (88.2%)	0.658± 0.13 (129.5%)
	30	0.519 ± 0.14	0.464 ± 0.11 (89%)	0.638 ± 0.13 (122.9%)	0.475 ± 0.13	0.407 ± 0.12 (86%)	0.656 ± 0.14 (138%)
Cell doubling time, t_d (day)	6	1.5 ± 0.09	1.3	1.1	1.4 ± 0.12	1.5	1.1
	30	1.3 ± 0.14	1.5	1.1	1.5 ± 0.13	1.7	1.1

The number in parentheses represents the percentage value in relative to the 1st passage, if the 1st passage considered as 100% [a]Mean ± SD (n = 3).

conditions after 11 passages. Continued passage of all pools in the absence of zeocin did not result in a weakening or loss of reporter gene expression.

The activity of β-gal expression at the end of long term culture was compared in both parental clones TF8(1) and TF9(7). The β-gal activity of TF8(1) sub-clone in the presence of zeocin after 11 passages decreased around 50% compared to the activity of its initial value for both culture volumes (Table 2). However, no significant change in specific activity was observed during long term culture for sub-clone TF9(7) at the present of zeocin except a decline by only 2% in 30 ml culture. The continuous presence of zeocin in culture medium did not increase the percentage of cells exhibiting β-gal expression. However, unexpected specific activity were observed when both of the clone TF8(1) and TF9(7) were incubated in the absence of selective marker, with productivity increased clearly over time. The β-gal productivity for subclone TF8(1) increased 71% when it was incubated in 6 ml culture volume and rapidly increased 100% up when expanded to 30 ml culture volume. Productivity of clone TF9(7) enhances 4-fold higher from its initial productivity after 11 passages in 6 and 30 ml culture volume medium without zeocin selection pressure. In terms of overall productivity upon long term culture in the presence and absence of zeocin selection pressure, clone TF9(7) was identified as one of the more stable clones. The β-gal productivity was up to 2-fold higher in the TF9(7) clone as compared to the TF8(1) clone in both selective and non-selective media for 30 ml culture volume.

To investigate whether clonal viability is related to the variation of changes in β-gal productivity of sub-clones during long term culture, viable cell concentration from TF8(1) and 9(7) at the beginning until the end of 11th passage was determined using hemacytometer. The cell concentration was counted on day 4 every passage. Table 2 shows the change in viable cell concentration of two

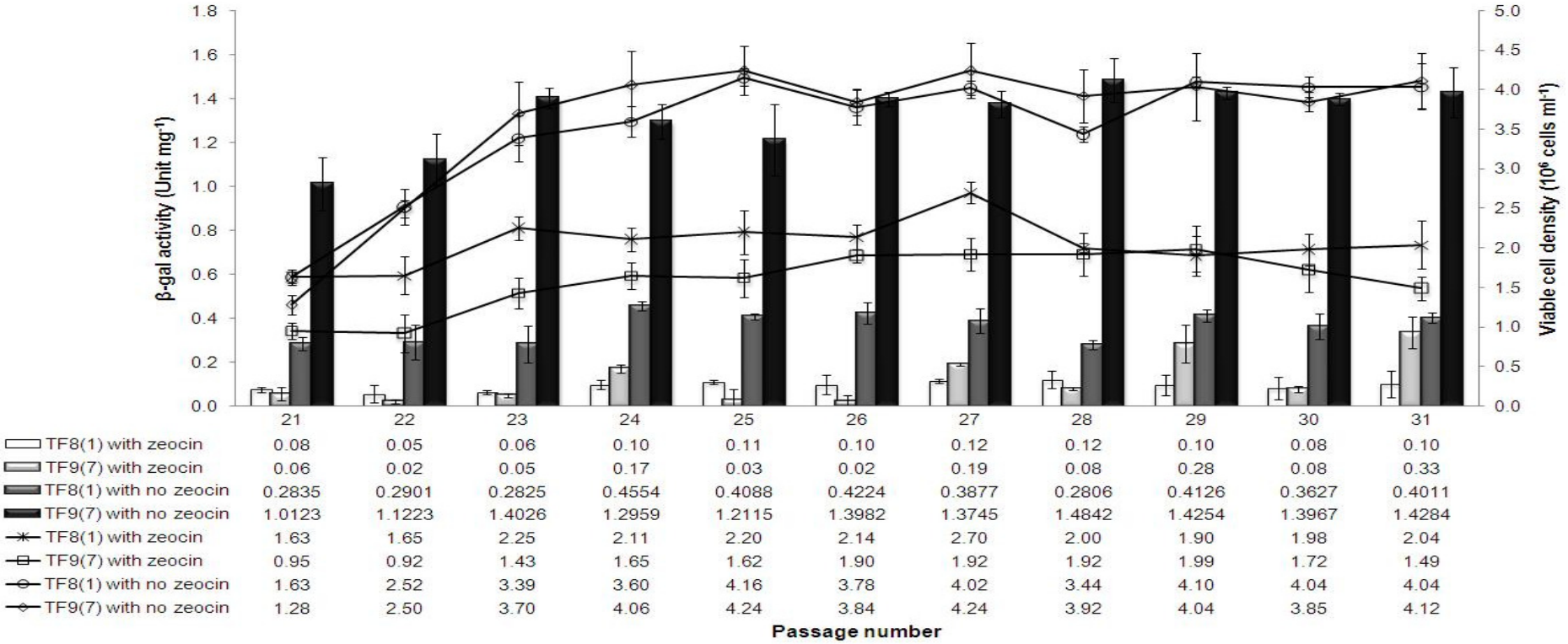

Figure 1. Changes in β-gal activity and viable cell density of clones TF8(1) and TF9(7) when long term cultured in the presence or absence of zeocin in 6 ml culture volumes. The error bars represent the standard deviations calculated from three independent experiments.

representative sub-clones. Both of the clones demonstrated increasing viable cell concentration in a range 3 to 25% over time when cultured in the presence of zeocin. Clones TF9(7) in non-selective medium reached a maximum viable cell concentration of 4.12×10^6 cell/ml, corresponding to 3-fold increase as compared to the initial value in 6 ml culture volume. However, the viable cell concentration of both clones in the absence of zeocin does not always increase with the increasing medium volume. However, cell growths were higher compared to the concentration in the presence of zeocin. When cultured without zeocin the viable cell concentration increased rapidly that maybe affected by the non stressful condition of number represents the mitochondrial activity of the *lac*Z-transfected cells in selective and non-selective medium. This shows that *lac*Z expression helps maintain the mitochondrial activity even under absence of selective pressure.

We observed that the β-gal productivity and cell growth rate remained relatively stable and increased rapidly when the cells were cultured in the absence of zeocin, except at the early passages of each clone (Figure 1 and Figure 2). The clones would require some time to recover to the new environment before the *lac*Z gene was amplified

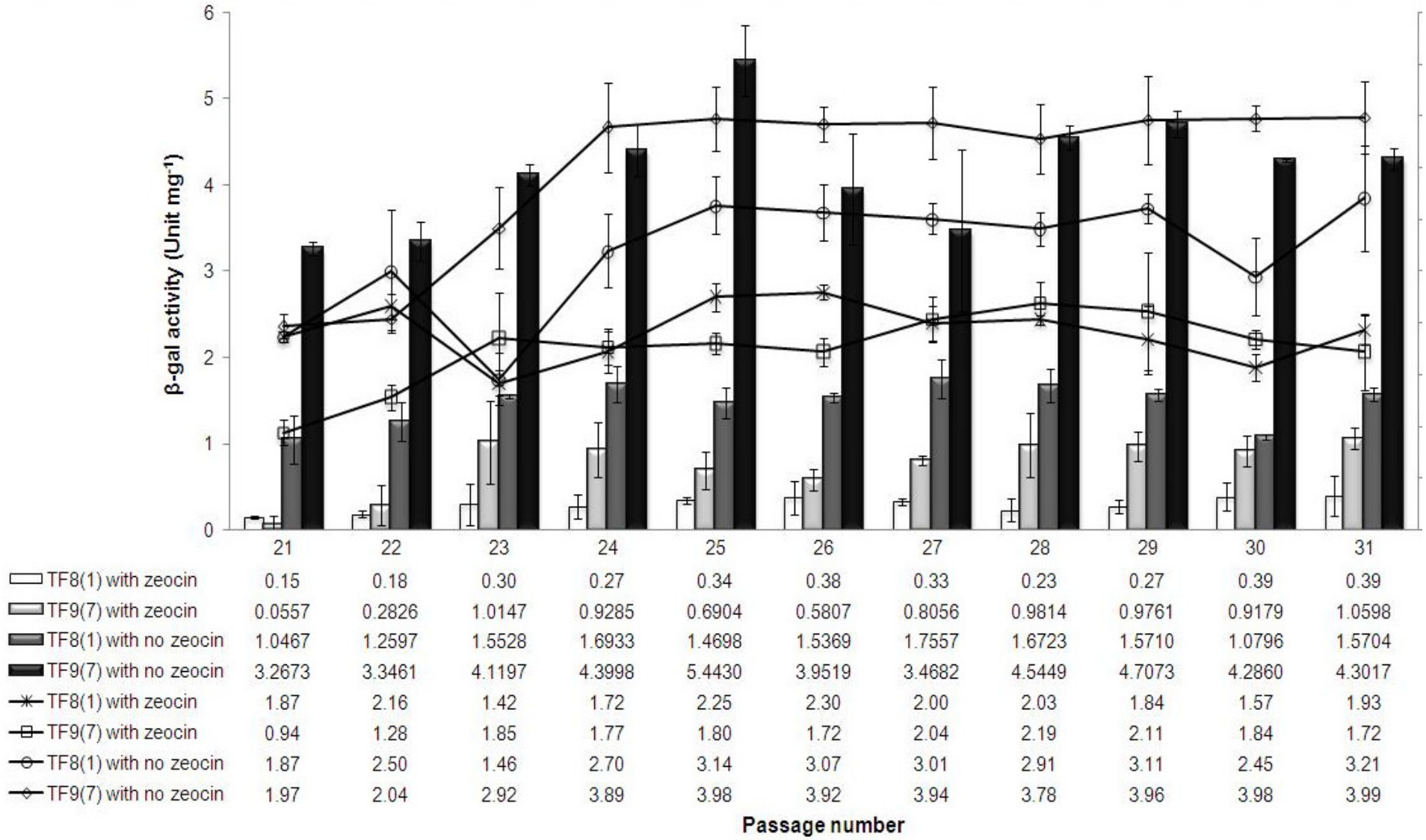

Figure 2. Changes in β-gal activity and viable cell density of clones TF8(1) and TF9(7) when long term cultured in the presence or absence of zeocin in 30 ml culture volumes. The error bars represent the standard deviations calculated from three independent experiments.

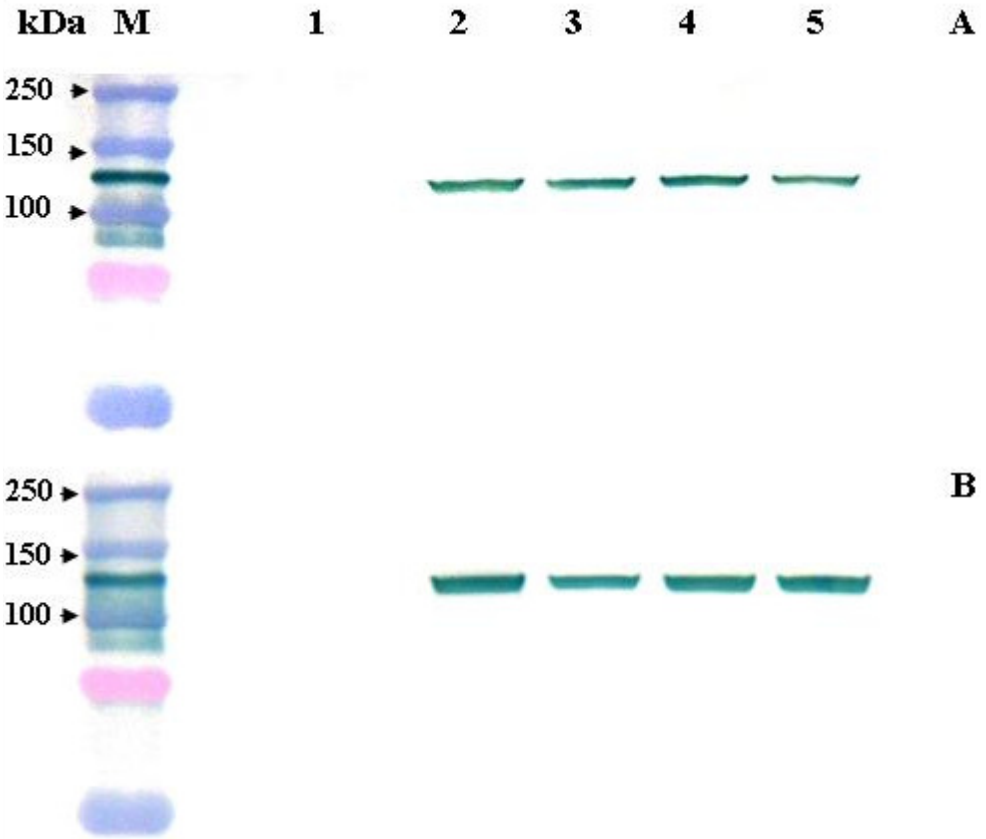

Figure 3. β-galactosidase expressions were verified using Western blot analysis. A: long-term culture in the presence of zeocin, B: long-term culture in the absence of zeocin. Band representing the β-galactosidase from the long term culture in the absence of zeocin showed bulkier line compared to the samples in the presence of zeocin at the same amount of total protein. Lane M: Protein marker; lane 1: Non transfected CHO cell; lane 2: TF9(7) cultured in 30 ml medium; lane 3: TF8(1) cultured in 30 ml medium; lane 4: TF9(7) cultured in 6 ml medium; lane 5: TF8(1) cultured in 6 ml medium.

stably in the medium without zeocin. All high producer clones included in the stability studies showed a variation ranging from 0.02 to 5.4430 unit/mg in specific productivity and variation in viable cell density from a range of 9.2×10^5 to 4.24×10^6 cells/ml over 11 passages when cultured with or without selective condition (Figures 1 and 2).

Table 2 shows the specific growth rate (μ) and cultivation time (t_d) of the sub-clones after long term culture under selective and nonselective growth conditions. No significant change in μ and t_d was observed at the end of the long term culture for both clones compared to initial rate in the present of zeocin. From overall observation, the specific growth rates for both clones were higher in the absence of zeocin compared to the presence of zeocin. Long term incubation for clone TF8(1) and TF9(7) in the nonselective medium showed increase in specific growth rate ranging from 24 to 52% in both culture volume with cell doubling time of 1.1 day. There was no consistent correlation between the cell growth rate and the culture volume in both clones. The growth rates of TF8(1) and TF9(7) sub-clones improved during the long term culture in the absence of zeocin. This improvement in cell growth occurred probably as a result of increase in the gene expression. A reduction in specific growth rate however did not significantly affect the specific activity as observed from Table 2. For example the specific activity of TF9(7) clone in the presence of zeocin for 30 ml culture volume showed the same degree despite a 12% decrease in specific growth rate.

Evidence of the over-expression of β-gal in the both clones was obtained by Western blot (Figure 3). This assay showed the difference between clones TF8(1) and TF9(7) in the presence and absence of zeocin. Both clones cultured in the absence of zeocin displayed higher levels of β-gal compared when cultured in the presence of zeocin, which express the same level of β-gal protein with parental clones. Both of the clones showed expected

band sizes at 118 kDa. In non-transfected CHO cell as negative control, no expression of β-gal is seen.

DISCUSSION

In this study, cells lines were generated by transfecting CHO cells with β-gal expression vector followed by zeocin selection and single cell cloning. Removal of zeocin as the selective pressure after 11 passages in this study has increased the expression of β-gal and specific growth rate of transfected CHO cells. As in previous report, selective pressure is not requisite for durable reporter gene expression in populations of stable clones (Gubin et al., 1997). A genetic study by Kim and Lee (1999) has shown that the presence of the selective pressure promotes cytogenetic heterogeneity which is an undesirable feature for commercial production. After removal of zeocin, improved β-gal expression was demonstrated in two pools and interestingly both pools showed higher specific activity of β-gal expression and specific growth rate compared to initial culture except at the early passages. This phenomenon is equivalent to a study conducted by Mielke et al. (2000) who found that most cells were not able to survive in a period of 2 weeks before recovering to normal growth rates that indicate the cell is ready to express the recombinant protein.

The consistency and high expression of β-gal in the TF8(1) and TF9(7) sub-clones over 11 passages or about two months in cultures also indicates durability and stability of β-gal expression on the growth of clonal populations. This finding is similar to the trend showed by Gubin et al. (1997) which demonstrated that the percentage of GFP positive cells increased or maintain after removal of geneticin from the growth medium within 8 weeks. Kaufman et al. (1985) demonstrated that in some cells lines maintained stable expression for more than 30 cell doublings or passage in the absence of methotrexate (MTX), whereas in other clones, expression decreased significantly during the same time period. Several studies reported that production stabilized after an initial 40 to 70% decrease in productivity upon removal of MTX or puromycin selection pressure after 8 weeks cultivation (Derouazi et al., 2006; Kim et al., 1998). It is clear from the results that the absence of selective pressure did not disturb the stability of recombinant protein expression. Yoshikawa et al. (2000a, b) described that the phenomenon occurs due to amplified transgenes located near the telomeric regions of chromosomes. Instability has also been observed in the presence of zeocin in this study. The rapid β-gal expression decrease of 40 to 50% in TF8(1) sub-clones within 11 passages with zeocin was similar to that observed in the report by Fann et al. (2000), whose t-PA production rate declined in a range 35 to 40% after 14 weeks being cultured in MTX.

At the end of the 11th passage, μ and specific activity in the absence of zeocin was 1.4 and 4 times higher than that in the present of zeocin. A study by Chusainow et al. (2009) demonstrated the rapid μ increase when the producer clones were cultured without MTX as the selective pressure. However, due to a 71% decline in specific productivity, decreased antibody titer over 36 passages was noted. The combination of good growth and high specific productivity makes a cell line suitable for scale-up and manufacturing (Wurm, 2004). Morrison et al. (1997) observed no significant change in growth rate when clone 29.8.6 (GP63E265D) was grown in the presence and absence of MTX for 138 days. Observation by Yoshikawa et al. (2000a, b) found that the specific growth rate of high producer clones depends on the transgene integration sites in the CHO chromosomal.

There are several different antibiotic-based selection systems which have been used in stable transfection. The most commonly used selection methods are based on the dihydrofolate reductase (dhfr) and the glutamine synthetase (gs) genes (Wurm, 2004). However selection system also can be achieved by using genes which confer resistance to antibiotics such as G418, hygromycin B, zeocin, blasticidin or puromycin (Matasci et al., 2008). In this study, zeocin resistance gene was used as a selection system in CHO cell expressing β-gal protein. Zeocin is a member of the bleomycin/phleomycin family of antibiotics, resistance to zeocin is conferred by the *Sh ble* gene, which known to encode a protein that binds to the antibiotic and prevents it from binding DNA (Trastoy et al., 2005). One factor which affected the transfection is the choice of vector promoter. The pcDNA4/HisMax-TOPO/*lac*Z plasmid with a CMV promoter was found to be a suitable reporter for stable transfection in CHO cell. According to Delacôte et al. (2007) the need to transfect with a zeocin resistance gene using the same vector might be a disadvantage of this plasmid because it may fail to detoxify cells completely and results in selection to chronic sublethal numbers of DNA double-strand breaks. However, this did not affect the frequency with which the cells developed zeocin resistance and stable long term expression of β-gal. The small size of the zeocin resistant gene (378 bp) also keeps the overall size of the pcDNA4 vector small and gives advantage for high transfection efficiencies (Bennett et al., 1998). This study showed major advantage of the pcDNA4/HisMax-TOPO/*lac*Z plasmid because of its stable integration and strong expression of β-gal in the CHO cell. The stability of both clones was confirmed by continuous expression of β-gal even when zeocin was removed from the 21st passages. Stable integration of pcDNA4/HisMax-TOPO/*lac*Z plasmid in these CHO cell lines led to a continuous cell expression of β-gal in the prolonged absence of a selection agent. This study also indicates that the stably integrated *lac*Z gene is not subject to transcriptional silencing over time in culture.

In conclusion, we demonstrated a key important for

long term, durable and high expression of β-gal in mammalian cells culture without additional antibiotic selection. Therefore, we suggest β-gal itself may be adequate as a marker of durable transfection and as machinery for monitoring transgene expression levels in cloned populations of mammalian cells.

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