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COMPARISON OF MULTICOPY PRO-MICROBIAL TRANSGLUTAMINASE ENCODED GENE EXPRESSION IN *PICHIA PASTORIS*

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Transglutaminases catalyse the formation of an isopeptide bond between the group of γ-carboxamides of glutamine residues and primary amine groups of proteins. It is widely used in different food industries in dairy, meat, and bakery products. In this work, the effect of the copy number of gene expression cassette on the extracellular production of pro-MTGase under the *GAP* promoter in *Pichia pastoris* was elucidated. Expression vector carrying the *Streptomyces mobaraensis* pro-MTGase encoded gene was constructed and transformed into the *P. pastoris* X33. The production of pro-MTGase in single copy and three copies expression cassettes containing clones were compared under the same fermentation conditions. More than 30% enzyme activity was obtained from single copy expression cassette containing clone compared to three copies expression cassettes containing clone. Besides, the amount of the enzyme produced per cell was found to be 24% higher in the fermentation broth of single copy expression cassette containing clone. As a conclusion, there is an inverse correlation between the extracellular production of pro-MTGase and the copy number of gene expression cassette.

Keywords: pro-MTGase, *GAP* promoter, gene copy number, *Pichia pastoris*

Transglutaminases (TGase) catalyse the acyl transfer reactions and they can change the properties of proteins by means of amine incorporation, crosslinking, and deamidation reactions. They are frequently used in food industry as a binding agent in restructured meat products (Uresti et al., 2004; Carballo et al., 2006). TGases improve quality and texture parameters of dairy products (Rossa et al., 2012; Damodaran & Agyare, 2013). It is also used in the food industry for increasing the volume and improving the texture of bakery products (Seravalli et al., 2011). Beside food industry, TGases are used in biomedical engineering, textile and leather industries. Thus, the increasing need for TGases at industrial scale has led scientists to study recombinant production of this enzyme. Studies on recombinant production of TGase studies using *E. coli*, *Pichia pastoris (Komagataella phaffii)*, and *Candida boidinii* as expression system have been published (Yurimoto et al., 2004; Sommer et al., 2011; Li et al., 2013; Salis et al., 2015).

In recent years, methylotrophic yeast *P. pastoris* has become the most popular expression system for recombinant protein production. It is capable of many post-translational modifications like proteolytic processing, disulfide bridge formation and glycosylation. In addition to these properties, it has inducible and constitutive promoters, grows rapidly in an inexpensive media, and is available as commercial strain and vector. All these properties make *P. pastoris* expression system more advantageous than other yeast and bacterial expression systems.

Several factors such as codon usage, gene copy number, secretion signal peptide, strong promoter, and bioreactor conditions affect the efficiency of expression of recombinant

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proteins. These factors are independent of each other, but researchers should find the correct combinations of them to increase yield expression as all these factors are protein specific. High gene copy number is known to be one of the most important factors that increase protein expression yield (Vassileva et al., 2001; Norden et al., 2011). Some studies show that high gene copy number transformants have lower protein expression efficiency (Hohenblum et al., 2004; Dagar & Kasha, 2018).

The aim of this study was to compare the yield of recombinant pro-MTGase production between transformants having single copy and three copies expression cassettes in *P. pastoris* expression system.

1. Materials and methods

1.1. Strains, plasmids, and media

Restriction enzymes, ligase, DNA and protein markers used in this study were obtained from Thermo Fisher Scientific. Cultivation media constituents were purchased from Becton Dickinson and Company. Other chemicals and reagents were of analytical grade and acquired from Sigma-Aldrich, Merck. The yeast expression vector pGAPZαA was obtained from Invitrogen. *E. coli* XL1-Blue cells were used for cloning. Wild-type *P. pastoris* X33 strain was used for protein expression.

1.2. Construction of expression vector

pGAPZαA vector and pro-MTGase gene (1131 bp, UniProtKB-P81453) were digested with the *Xho*I and *Xba*I restriction enzymes, ligated by using Rapid DNA Dephos & Ligation kit, and transformed into the competent *E. coli* XL1Blue cells. Expression vector was confirmed by restriction enzyme digestion and DNA sequencing.

pGAPZα-pro-MTGase plasmids were linearized with *Avr*II and transformed into the component *P. pastoris* X33 strain by the electroporation method. Transformant X33 cells were grown on YPD agar plate containing 100 mg l^{-1} and 500 mg l^{-1} zeocin.

1.3. Southern blot analysis

Genomic DNA was isolated from yeast strains using MasterPureTM Yeast DNA Purification Kit, and concentrations were measured with Qubit dsDNA BR assay kit. For Southern blot analysis, 1 μg of DNA was digested with *Cla*I restriction enzyme and digested DNA was separated on 0.8% TAE agarose gel by electrophoresis. DNA was transferred to positively charged nylon membrane. Then the DIG-labelled *GAP* promoter probe was hybridized with the membrane. DIG-High Prime DNA Labeling and Detection Starter kit II was employed for scanning the chemiluminescence signal on X-ray films.

1.4. Pro-MTGase production of the Pichia pastoris *expression system at a bioreactor scale*

A 5 l bioreactor (Sartorius Stedim BIOSTAT B) was employed to produce proMTGase at a large-scale. One millilitre of pGAPZα-pro-MTGase frozen culture was inoculated into 100 ml BMGY and incubated at 28 °C for approximately 20 h (at about 10 OD_{600nm}). This culture was used as inoculum for 2 l citric acid media (2.0 g l^{-1} citric acid monohydrate, 45.6 g l^{-1}

glycerol (86%), 12.6 g l^{-1} $(NH4)_2HPO_4$, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.9 g l^{-1} KCl, 0.022 g l^{-1} $CaCl_2 \cdot 2H_2O$). After autoclaving, pH was adjusted to 5 with 25% HCl and 4.6 ml l^{-1} PTM1, then 2 ml l^{-1} biotin (0.2 g l^{-1} stock) was added to this media. Batch phase lasted about 20 h and was completed with dissolved oxygen (DO) spike. This sudden rise in DO is indicative of the depletion of glycerol in batch phase media. After this carbon-exhaustion signal, the fed-batch phase of fermentation was started by feeding 50% (w/v) aqueous glucose solution. The feed rate of glucose solution was exponentially increased to keep specific growth rate (μ) constant at 0.02.

The temperature and pH during the batch phase were maintained at 28 °C and 5.0, respectively. At the beginning of the fed-batch phase, the temperature was set to 20 °C shortly after the DO spike, and the pH was linearly increased from 5 to 7 in the first 2 h. Fermentation pH value was controlled by 26% (v/v) NH_4OH which, also served as nitrogen source. DO level was kept at 20% saturation with agitation and by adding air (1.5 vvm) and pure oxygen as necessary.

1.5. Total protein determination

The total protein concentration of supernatants was determined with Coomassie Plus – The Better Bradford Assay Kit. Bovine serum albumin was used as standard, and absorbance was measured at 595 nm by a spectrophotometer.

1.6. MTGase activity assay

Before measuring enzyme activity, pro-MTGase was activated with dispase I, as previously described (Sommer et al., 2012). After activation, MTGase activity was measured by the colorimetric hydroxymate method (Folk, 1969). The 200 μl reaction cocktail (containing Z-Gln-Gly, 1M Tris buffer pH 6, and 200 mM hydroxylamine 20 mM reduced glutathione mixture) was incubated at 37 °C. Then 30 μl of activated supernatant samples were added into the reaction cocktails and incubated at 37 °C for 10 min after mixing by inversion. At the end of 10 min, 1 ml stop solution (1 volume 3 M HCl, 1 volume 12% TCA, and 1 volume 5% $FeCl_3$) was added and mixed in by inversion. The stopped reaction mixture was centrifuged at 4000 *g* for 5 min, and then transferred into a cuvette for measuring the enzyme activity at 525 nm. In addition to this, the activated supernatant and the reaction cocktail were mixed, and the reaction was stopped immediately to prepare the test blank tube. The standard tubes contain 100 μl L-glutamic acid-γ-monohydroxymate and 1 ml stop solution, and the standard blank tube contains 100 μl water instead of L-glutamic acid-γ-monohydroxymate. Test blank, standards, and standard blank were centrifuged for 5 min and absorbances were measured without incubation at 37 °C. The enzyme activity was expressed in U ml^{-1} and 1 unit was defined as 1 μmole of hydroxamic acid formed per minute under the given conditions.

1.7. SDS-PAGE analysis

The supernatant samples were mixed with 4X SDS-PAGE loading buffer (200 mM Tris-Cl pH 6.8, 8% SDS, 0.4% bromophenol blue, 40% glycerol) and 1 M dithiothreitol (DTT) and then incubated at 70 °C for 10 minutes. After incubation, samples were separated by 10% SDS-PAGE gel by using 1X Tris-Glycine-SDS buffer. Gel stained with Coomassie blue was scanned with Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). Page Ruler unstained protein ladder was used as molecular weight marker.

2. Results and discussion

pGAPZα-proMTGase expression cassette was constructed and transformed into *P. pastoris* X33. The copy number of pGAPZα-proMTGase expression cassette inserted in the genome was identified by Southern blot analysis (Fig. 1). The single copy and three copies expresssion cassettes containing clones were selected for bioreactor scale pro-MTGase production.

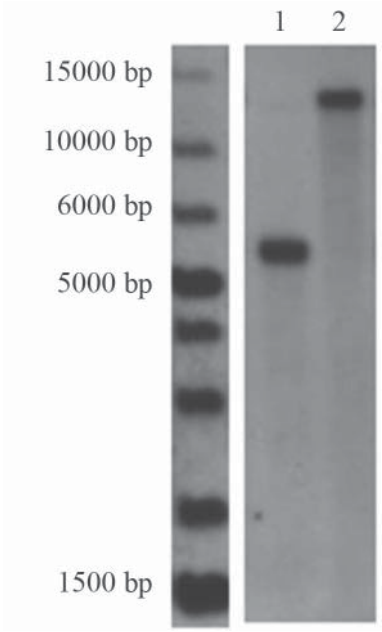

Fig. 1. Southern blot analysis to determine gene copy number of expression strains. Genomic DNA of clones was digested with *Cla*I restriction enzyme, separated in 0.8% agarose gel and transferred to positively charged nylon membrane for hybridization with DIG-labelled probe. Expected band sizes were 5800 bp for single copy clones and 14296 bp for three copies clones. 1: single copy expression cassette containing clone, 2: three copies expression cassette containing clone

Two-step fermentation strategy was applied to each clone for protein production. During the first step, glycerol batch phase, cells use the glycerol as a carbon source to increase cell concentration. At the end of batch phase, wet cell weight (WCW) was increased up to 133.4 g l^{-1} and 137.5 g l^{-1} for single copy expression cassette and three copies expression cassette containing clones, respectively. DO-spike strategy was used to pass the second stage of fermentation, namely, glucose fed-batch. In this phase, cells were fed with 50% glucose solution to keep specific growth rate (μ) constant at 0.02, and it was completed in 71 h. The highest WCW concentrations were obtained as 339.7 g l^{-1} and 310.5 g l^{-1} after 71 h cultivation for single copy expression cassette and three copies expression cassette containing clones, respectively (Fig. 2). While the concentration of the cells was measured from 1 ml culture, the enzyme activity and total protein concentration analysis were performed in the supernatant obtained from 1 ml of culture precipitation. Therefore, the total values of protein concentration and enzyme activity in the supernatant were re-calculated by applying the correction factor

as given below to the protein concentration (Eq. 1) and enzyme activity (Eq. 2) values measured in the supernatant.

$$Tp(t) = Tp^{*}(t) \times (1 - \frac{x(t) \times \sigma}{\rho}) \quad (1)$$

$$P(t) = P^{*}(t) \times (1 - \frac{x(t) \times \sigma}{\rho}) \quad (2)$$

In these equations, * shows total protein concentration or enzyme activity measured in supernatant, x(t) is cell concentration at time t; σ is dry cell fraction of wet cell weight 0.28 $g_{dcw}\, g_{wcw}^{-1}$; ρ is the cell density of *P. pastoris* 330 $g_{dcw}\, l^{-1}$. As shown in Figure 2A, total protein concentration and WCW values of the two clones were a little bit different from each other. It was observed that cell concentration as well as extracellular protein concentration increased during the 71 h for both clones.

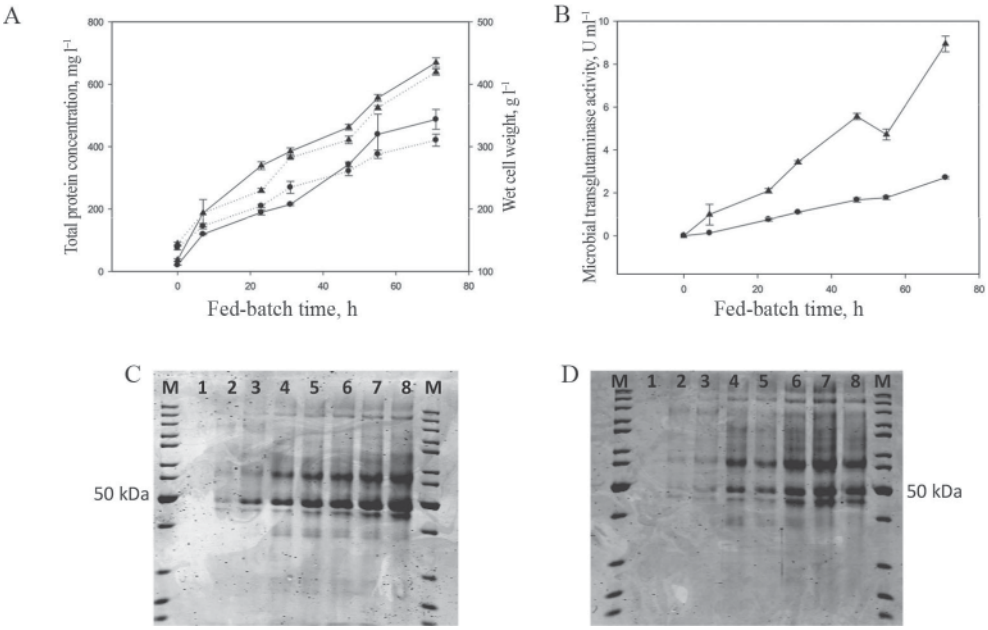

Fig. 2. Time-course of pro-MTGase expression in fed-batch cultures. A) Amount of total protein, mg l^{-1} and wet cell weight, g l^{-1}, B) Microbial transglutaminase activity, U ml^{-1}. The error bars represent standard deviation of three runs. C) and D) SDS-PAGE analysis of pro-MTGase expression under *GAP* promoter in bioreactor scale experiments of 1 and 3 copies expression cassette containing clones, respectively. The expected molecular weight of pro-MTGase is 47 kDa. M: protein marker; 1: batch time end; 2: fed-batch 0; 3: fed-batch 7; 4: fed-batch 23; 5: fed-batch 31; 6: fed-batch 47; 7: fed-batch 55; 8: fed-batch 71 samples.
A): —▲—: Total protein concentration for 1 copy; —●—: Total protein concentration for 3 copies; - -▲- -: Wet cell weight for 1 copy; - -●- -: Wet cell weight for 3 copies
B): —▲—: MTGase activity (U ml^{-1}) for 1 copy expression casette; —●—: MTGase activity (U ml^{-1}) for 3 copies expression casettes

On the other hand; at the end of fermentation, the MTGase enzyme activity measurement showed that the single copy expression cassette containing clone (8.9 U ml^{-1}) had higher enzyme activity than the 3 copies expression cassette containing clone's activity (2.7 U ml^{-1})

(Fig. 2B). According to the SDS-PAGE results, the accumulation of pro-MTGase enzyme in the culture supernatant increased during the fed-batch time for each clone (Figs 2C, 2D). Added amount of glucose (g) and specific glucose consumption rate (qs, $g_{glu}\ g_{dcw}^{-1}\ h^{-1}$) of fermentation process of each clone are presented in Figure 3. It is seen that the qs value of each fermentation was consistent with the specific growth rate (μ=0.02) and remained constant during the fermentation. To obtain constant μ (0.02), mean qs values were calculated as 0.06 $g_{glu}\ g_{dcw}^{-1}\ h^{-1}$ for single copy and three copies expression cassette containing clones. Total product (U), specific enzyme activity (U mg^{-1}), production rate (rp; U h^{-1}), specific production rate (qp; U $g_{dcw}^{-1}\ h^{-1}$), and volumetric production rate (Qp; U $h^{-1}\ l^{-1}$) parameters of bioreactor scale pro-MTGase production with single copy and three copies expression cassette containing clones are summarized in Table 1. After 71 h fed-batch phase, the specific activity of MTGase in fermentation broth was 13.35 U mg^{-1} and 5.57 U mg^{-1} for single copy and three copies expression cassette containing clones, respectively. Specific production rate of fed-batch phase was found to be 3.82 U $g_{dcw}^{-1}\ h^{-1}$ for single copy expression cassette containing clone and 0.92 U $g_{dcw}^{-1}\ h^{-1}$ for three copies expression cassette containing clone. In addition to this, the volumetric production rate of single copy expression cassette containing clone (356.87 U $h^{-1}\ l^{-1}$) was higher than that of three copies expression cassette containing clone (84.21 U $h^{-1}\ l^{-1}$). All these results showed that containing three copies expression cassette does not positively affect the production of pro-MTGase. Although the same fermentation conditions were applied for each clone, it was observed that single copy expression cassette containing clone produces much more enzyme.

Table 1. Comparisons of total protein, total product, specific activity, production rate, specific production rate, and volumetric production rate of bioreactor scale pro-MTGase expression between 1 copy and 3 copies expression cassettes containing clones

Gene copy number	Fermentation time (h)	Total protein (mg)	Total product (U)	Specific activity (U mg^{-1})	Production rate, rp (U h^{-1})	Specific production rate, qp (U $g_{dcw}^{-1}\ h^{-1}$)	Volumetric production rate, Qp (U $h^{-1}\ l^{-1}$)
1	7	385	2028	5.27	393	4.30	190.8
	23	760	4695	6.18	192	1.53	85.8
	31	898	7991	8.90	195	1.33	83.7
	47	1229	14776	12.02	407	2.02	153.1
	55	1567	13291	8.48	616	2.60	218.6
	71	2327	31071	13.35	1240	3.82	356.9
3	7	245	260	1.06	123	1.29	59.9
	23	427	1708	4.00	87	0.69	38.6
	31	507	2570	5.07	87	0.60	37.0
	47	913	4441	4.86	124	0.64	46.7
	55	1238	4995	4.03	161	0.72	57.0
	71	1561	8700	5.57	270	0.92	84.2

P. pastoris is the most popular expression system to produce recombinant protein. To meet the enzyme demand of industry, high-level recombinant production of enzymes has crucial importance. Several factors affecting protein production such as codon usage, gene

copy number, secretion signal peptide, strong promoter, bioreactor conditions should be optimized to increase the yield of production. Therefore, the aim of this study was to determine the effect of gene copy number on the high-level extracellular production of pro-MTGase enzyme in *P. pastoris*.

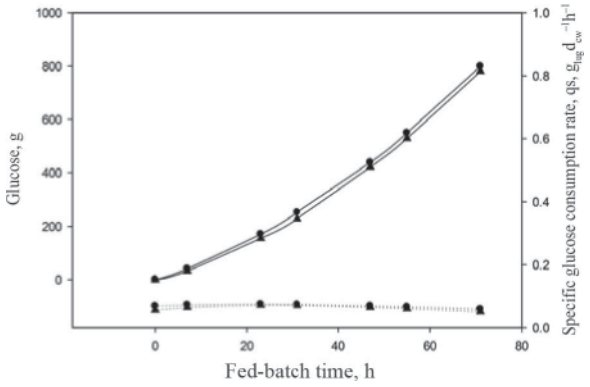

Fig. 3. Amount of glucose (g) added to fermentation medium during 71 h fed-batch phase and specific glucose consumption rate (qs) of bioreactor scale expression of pro-MTGase enzyme.
—▲—: Added glucose for 1 copy; —●—: Added glucose for 3 copies; - -▲- -: qs for 1 copy; - -●- -: qs for 3 copies

In the literature, there are some studies showing that productivity increases with increasing gene copy number (Clare et al., 1991; McGrew et al., 1997; Vassileva et al., 2001; Norden et al., 2011). On the contrary, it has been reported in studies that high gene copy number negatively affects the protein expression in *Pichia* system (Hohenblum et al., 2004; Dagar & Kasha, 2018). Hohenblum and co-workers (2004) obtained high trypsinogen yield with 1 and 2 copies expression cassette containing clones. However, they observed that the productivity of clones having 3 or more expression cassettes decreased. Similar results were also reported by Dagar and Kasha (2018). According to their study, the expression of human interleukin-3 decreased in clones with more than 8 copy expression cassettes.

In this work, 1 and 3 copies clones were compared for production of pro-MTGase enzyme under the control of *GAP* promoter of *P. pastoris* in bioreactor scale. Similar results were obtained by Hohenblum and co-workers (2004) and Dagar and Kasha (2018). It was observed that, the productivity of expression is higher in single copy expression cassette containing clone than three copies expression cassette containing clone. The most important reason of this situation have been known as disruptions of post-translational modifications, such as disulfide bridge formation, folding in endoplasmic reticulum (ER), and signal sequence processing (Macauley-Patrick et al., 2005; Shen et al., 2012). Because of these reasons, the excess proteins produced cannot get out of the cell and remain within the cell (Hohenblum et al., 2003).

When the proteins are not folded properly, they accumulate in the ER and induce unfolding protein response (UPR) by triggering the transcription of stress responsive genes encoding ER resident chaperone binding protein (BiP, also known as Kar2) (Hohenblum et al., 2004). Hohenblum and co-workers (2004) reported that BiP was expressed constitutively during the protein expression under the regulation of *GAP* promoter. In this study, pro-

MTGase was also expressed under the control of *GAP* promoter. As a result, UPR observed in previous studies also dominated the results.

3. Conclusions

In this report, the effect of gene copy number on pro-MTGase expression in *P. pastoris* was determined. According to the results of the fed-batch fermentation in bioreactor, single copy expression cassette containing clone could be more effectively used for the expression of recombinant pro-MTGase enzyme production when compared to three copies expression cassette containing clone.

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Abbreviation list

Pro-MTGase: pro-microbial transglutaminase
TGase: Transglutaminase
GAP: Glyceraldehyde-3-phosphate dehydrogenase
E. coli: Escherichia coli
P. pastoris: Pichia pastoris
S. mobaraensis: *Streptomyces mobaraensis*
DO: Dissolved oxygen
WCW: Wet cell weight
DTT: Dithiothreitol
DCW: Dry cell weight
qs: Substrate consumption rate
μ: Specific growth rate
U: Total product
U mg^{-1}: Specific enzyme activity
rp: Production rate
qp: Specific production rate
Qp: Volumetric production rate
ER: Endoplasmic reticulum
UPR: Unfolding protein response
BIP: Chaperone binding protein