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Enhancement of transglutaminase production in *Streptomyces mobaraensis* DSM 40587 by non-nutritional stress conditions: Effects of heat shock, alcohols, and salt treatments

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Abstract−Stress-mediated bioprocess is a strategy designed to enhance biological target productivity. In this study, an attempt was made to enhance transglutaminase (TGase) production by *Streptomyces mobaraensis* by using different stress conditions including heat shock, alcohols and salt stress. Results showed that the effects of stress on TGase production depended on the type applied. For heat shock, TGase production (1.32 U/ml) was recorded maximum in the culture treated at 48 h post inoculation in water bath at 60 °C for 1 min. For alcohols treatment, the maximum activity of TGase (1.77 and 1.75 U/ml) was obtained when 3% methanol was added into the medium at 0 or 24 h of fermentation. However, a 3.5-fold increased production of TGase (3.8 U/ml) was observed in the medium supplemented with 0.2 mol/*l* $MgCl_2$ compared with the basic medium at the beginning of fermentation. In conclusion, TGase production from *S. mobaraensis* was improved by heat shock, methanol and salt stress treatments, $MgCl_2$ stress was the most effective.

Key words: *Streptomyces mobaraensis*, Transglutaminase Production, Heat Shock, Salt Stress, Alcohols Treatment

INTRODUCTION

Transglutaminase (TGase, protein-glutamine: amine γ-glutamyl-transferase, EC 2.3.2.13) is a family of enzymes that can catalyze acyl transfer reaction using peptide-bond glutamine residues as acyl donors and several primary amines as acceptors for modification of functional properties [1-5]. It can be produced by some *Streptomyces* species [1], *Bacillus* [6] and yeast [7].

For the development of a commercially feasible fermentation process, improvement in TGase yield and overall productivity is essential. The industrial potential of TGase has stimulated research in the development of methods for improving the fermentation medium or condition [8-12], since the production level of TGase in original medium is sometimes low for commercial exploitation. So the mechanisms of TGase production and method improvement for productivity are important themes.

In a variety of *Streptomyces*, growth suppression due to exhaustion of carbon, nitrogen, or phosphate from the culture medium often allows for expression of the genetic information for secondary metabolites. A stress-mediated bioprocess was another designed strategy to enhance biological target productivity [13,14]. The nutritional status of the environment switch superimposed on the pathway-specific control mechanisms and remarkable onset of secondary metabolism [15]. Many antibiotic producing microorganisms have been documented as generating the secondary products only by heat shock, ethanol treatment and salt stress. Doull et al. [16] reported that the synthesis of jadomycin B, a polyketide antibiotic, could be promoted by heat shock or ethanol treatment. Nakata et al. [17] observed that the stress imposed by a continuous feed of high ethanol, high NaCl concentration, or a high temperature shock, increased antibiotic production by several times in *Pseudomonas fluorescens* S272. Himabindu and others [18] found that application of different stress conditions like heat shock, high ethanol and high NaCl stress during fermentation was effective in gentamicin production. However, there is no report about promoting TGase production under stress-mediated conditions.

In this study, effects of heat shock in water bath, high NaCl concentration and high alcohols concentration during fermentation on enhancement of TGase production by *S. mobaraensis* were investigated. In addition, stress-mediated conditions were optimized.

EXPERIMENTAL SECTION

1. Bacterial Strain and Culture Conditions

S. mobaraensis DSM 40587 (DSMZ, Braunschweig, Germany) was selected as a TGase producer. For the formation of spores, an inoculum was spread on agar slants containing malt extract. After cultivation at 30 °C for 6-8 days, spores were collected in 5 ml of a sterile 0.1% aqueous solution of Tween 80 by scraping the surface of the medium with an inoculating loop. Spores from fresh cultures on agar were inoculated into 100 ml seed culture medium in 500 ml flasks in an orbital shaker (SPX - 150 C, Boxun, China) at 180 rpm and cultivated at 30 °C for 48 h. Up to 10% (volume) of this preinoculate was transferred to 50 ml basic culture medium in 250 ml Erlenmeyer flasks and incubated at 30 °C, 180 rpm. The seed culture medium was composed of 2% polypeptone, 0.2% K_2HPO_4, 0.2% KH_2PO_4 and 0.2% $MgSO_4 \cdot 7H_2O$; pH was adjusted to 7.0 with

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1 mol/*l* NaOH before autoclaving (0.1 MPa, 15 min). The basic culture medium (pH 7.0) was composed of 3% polypeptone, 1% soluble starch, 1% fructose, 0.2% K_2HPO_4 and 0.1% $MgSO_4 \cdot 7H_2O$.

2. Stress Methods

For heat shock treatment, the cultures were exposed to different temperatures of 50, 60 and 70 °C for 1 min under water bath and then cooled quickly back to 30 °C at 0, 24, 48 and 72 h after inoculation. TGase production, concentration of protein and growth of *S. mobaraensis* were estimated at 96 h of fermentation.

To test the effects of different alcohols on the production of TGase, cultures were supplemented with 1, 2 and 3% of ethanol and methanol at the beginning of fermentation. TGase production, concentration of protein and growth of *S. mobaraensis* were measured at 96 h of fermentation. To further test the condition of methanol stress on TGase productivity, cultures were supplemented with different concentrations of methanol (1, 3 or 5%) at 0, 24, 48 and 72 h post inoculation.

Five levels of sodium chloride (0.05, 0.10, 0.15, 0.20 and 0.25 mol/*l*) were added to 50 ml of basic fermentation medium at the beginning of fermentation, TGase production, concentration of protein and growth of *S. mobaraensis* were recorded at 96 h of fermentation. Further, sodium chloride was added into cultures at optimal level during the fermentation at 24 h interval (up to 72 h), TGase production and growth of *S. mobaraensis* were recorded at 96 h of fermentation.

To investigate effects of salts on TGase production, eight different salts including $MgCl_2$, NaCl, KCl, Na_2SO_4, $C_6H_5Na_3O_7$ (sodium citrate), Na_3PO_4, CH_3COONa and $CaCl_2$ were added into basic media at 0.2 mol/*l* and then autoclaved, with a basic medium serving as the control. Biomass and TGase level were determined and compared for all cultures at 96 h after of fermentation.

3. Dry Cell Weight Determination (DCW)

Dry cell weight was measured by filtering fermentation of *S. mobaraensis* through a pre-weighed filter paper (Whatman, GF/C). The residue on the filter paper was washed with distilled water and dried at 105 °C until a constant weight was obtained [9].

4. TGase Activity Assay

Activity of TGase was measured by using the colorimetric method [19], in which N-carboxybenzoyl-L-glutaminyl-glycine (Sigma, Shanghai, China) was used as the substrate. A calibration curve was obtained by using L-glutamic acid γ-monohydroxamate (Sigma, Shanghai, China). One unit of TGase was defined as the amount which caused the formation of 1.0 μmol of hydroxamic acid per minute at 37 °C. TGase Specific activity was expressed as units per milligram of protein.

5. Protein Concentration Assay

Protein concentration was determined by the method of Bradford [20], using bovine serum albumin (Sigma, Shanghai, China) as standard.

6. Data Analysis

All experiments were performed in triplicate independently, and analyses were carried out in duplicate. Statistical analyses were conducted by using the SPSS 14.0 for Windows (SPSS Inc.; Chicago, IL, USA). One-way ANOVA with Duncan's post-test was used. A probability level of $P<0.05$ was used throughout this study. Data were expressed as mean values±standard deviation (S.D.).

RESULTS AND DISCUSSION

1. Effects of Heat Shock on TGase Production

A general finding is that production of TGase rarely occurs during periods of rapid growth in rich media; rather, the onset of their biosynthesis generally coincides with periods of growth-rate reduction following exhaustion of carbon, nitrogen, or phosphate [1]. In our investigation, the fermentation liquid of *S. mobaraensis* was treated with heat shock at different temperatures As shown in Table 1, the fermentation liquid treated with lower temperature (50 °C) at any time enhanced the TGase biosynthesis significantly ($p<0.05$) but did not affect the cell growth. Growth of *S. mobaraensis* cell was significantly ($p<0.05$) reduced by being treated with heat at 60 and 70 °C compared with that of the control (cells were incubated at 30 °C without any treatment). However, when the treatment was performed at 72 h post inoculation, there was no effect on cell growth. This result implied that the high temperature stress during cell growth periods was severe on the growth of *S. mobaraensis* cell. Suitable

Table 1. Effects of heat shock period and temperature on TGase production and growth of *S. mobaraensis* DSM 40587

Time (h post inoculation)	Temperature (°C)	TGase activity (U/ml)	Protein concentration (mg/ml)	TGase specific activity (U/mg protein)	DCW (g/L)
Control	--	0.71 ± 0.04^{ab}	1.08 ± 0.14^{de}	0.66 ± 0.04^{a}	10.90 ± 0.07^{gh}
	50	0.92 ± 0.00^{cd}	1.10 ± 0.00^{de}	0.84 ± 0.01^{ab}	10.55 ± 0.19^{gh}
0	60	0.85 ± 0.01^{bc}	0.78 ± 0.08^{abc}	1.10 ± 0.01^{abc}	5.00 ± 0.23^{b}
	70	0.61 ± 0.00^{a}	0.53 ± 0.27^{a}	1.32 ± 0.07^{bc}	3.53 ± 0.49^{a}
	50	1.06 ± 0.01^{de}	1.02 ± 0.05^{cde}	1.04 ± 0.06^{abc}	11.32 ± 0.29^{h}
24	60	0.67 ± 0.02^{ab}	0.59 ± 0.20^{ab}	1.20 ± 0.37^{abc}	8.36 ± 0.96^{d}
	70	0.66 ± 0.01^{ab}	0.67 ± 0.10^{ab}	0.99 ± 0.15^{abc}	4.82 ± 0.01^{b}
	50	1.13 ± 0.15^{e}	1.23 ± 0.01^{ef}	0.92 ± 0.12^{abc}	10.63 ± 0.15^{gh}
48	60	1.16 ± 0.20^{e}	0.77 ± 0.02^{abc}	1.51 ± 0.30^{c}	8.43 ± 0.86^{de}
	70	0.75 ± 0.02^{abc}	0.87 ± 0.02^{bcd}	0.86 ± 0.00^{ab}	6.43 ± 0.02^{c}
	50	1.10 ± 0.11^{de}	1.42 ± 0.06^{f}	0.78 ± 0.11^{ab}	10.15 ± 0.16^{fg}
72	60	1.15 ± 0.07^{e}	1.01 ± 0.10^{cde}	1.15 ± 0.18^{abc}	9.30 ± 0.06^{ef}
	70	0.79 ± 0.13^{abc}	1.04 ± 0.17^{cde}	0.78 ± 0.25^{ab}	9.12 ± 0.08^{de}

All data were expressed as mean values±S.D. (n=6). Values followed by different letters in the same column are significantly different ($p<0.05$)

heat treatment (at 60 °C after 48 h post inoculation) enhanced TGase biosynthesis. Optimum TGase production was obtained with the cultures treated at 48 h post inoculation in a water bath at 60 °C for 1 min. Higher temperature treatment (60 or 70 °C) inhibited the extracellular protein secretion at the period of cell growth which was caused by decrease of the cell growth; however, no inhibitory effect was observed at the aging period (72 h post inoculation). These results indicated that protein biosynthesis was associated with cell growth. TGase specific activity (1.51 U/mg protein) was 1.3 times higher than that of the control (0.66 U/mg protein). Although the mechanism whereby heat shock induced the production of TGase has not been elucidated, we suggested that it related to the demonstrated ability of this treatment to trigger a heat-shock response. There was some evidence, moreover, linking genetic control mechanisms which initiate secondary metabolite production with global regulatory networks which sense fluctuations in nutrient availability and adjust metabolic activities. There was a report about effects of environmental stress on the production of antibiotics by members of the bacterial *genus Streptomyces*. Ngo and others [21] found heat treatment affected the cell growth and chitosanase from *S. griseus*. In the cases of heat treatments (41 °C), the growth rates of *S. griseus* and chitosanase activity were respectively 1.2 and 1.8 times, respectively, higher than that obtained under the control condition.

2. Effects of Different Alcohols on TGase Production

These are a few studies on improvement production of some secondary metabolites by methanol or ethanol. Growth of *S. clavuligerus* NP1 in the presence of methanol or ethanol resulted in a marked increase in the production of cephalosporin(s) from penicillin G by resting cells [22]. Haq and others [23] tested alcohols as stimulating agents and found that 1.0% (v/v) methanol resulted in a maximum amount (90.0±2.2 g/*l*) of anhydrous citric acid from *Aspergillus niger* GCB-47 which was 1.96-fold higher than the control.

In the present study, various concentrations (1-3%) of ethanol or methanol were added into the culture at the beginning of fermentation of *S. mobaraensis*. As shown in Table 2, the growth of *S. mobaraensis* and protein biosynthesis were inhibited by supplementing with ethanol or methanol. However, TGase production was improved significantly ($p<0.05$) to 1.37-1.47 U/ml and 1.66-1.76 U/ml ($p<0.05$), compared with the control (0.73 U/ml). Furthermore, TGase activity produced by methanol was much higher than that by ethanol significantly ($p<0.05$), indicating that methanol was a superior alcohol stimulant for TGase.

To further test the optimal condition of methanol stress, various concentrations (1, 3 and 5%) of methanol were added to the shake

Table 2. Effects of TGase production and growth of *S. mobaraensis* DSM 40587 with methanol and ethanol stress at the beginning of fermentation

Treatment	TGase activity (U/ml)	Protein concentration (mg/ml)	TGase specific activity (U/mg protein)	DCW (g/L)
Control	0.73±0.08^{a}	1.12±0.01^{e}	0.65±0.07^{a}	11.60±0.06^{f}
1% Methanol	1.76±0.07^{e}	0.97±0.01^{c}	1.81±0.10^{c}	10.73±0.11^{de}
2% Methanol	1.66±0.26^{de}	0.88±0.00^{b}	1.88±0.29^{c}	9.44±0.17^{ab}
3% Methanol	1.73±0.02^{de}	0.78±0.02^{a}	2.23±0.08^{d}	9.20±0.06^{a}
1% Ethanol	1.47±0.01^{cd}	1.02±0.05^{d}	1.45±0.08^{b}	11.26±0.53^{ef}
2% Ethanol	1.37±0.04^{bc}	0.95±0.01^{c}	1.43±0.03^{b}	10.40±0.11^{cd}
3% Ethanol	1.41±0.04^{b}	0.90±0.00^{b}	1.31±0.05^{b}	9.99±0.31^{bc}

Alcohols were added just after (0 h) incubation. All data were expressed as mean values±S.D. (n=6). Values followed by different letters in the same column are significantly different ($p<0.05$)

Table 3. Effects of methanol concentration and addition period on TGase production and growth of *S. mobaraensis* DSM 40587

Time (h post inoculation)	Methanol addition (%)	TGase activity (U/ml)	Protein concentration (mg/ml)	TGase specific activity (U/mg protein)	DCW (g/L)
Control	0	0.67±0.09^{a}	1.18±0.07^{c}	0.56±0.04^{a}	11.20±0.21^{h}
	1	1.51±0.19^{de}	0.95±0.03^{abc}	1.60±0.25^{cd}	8.57±0.23^{c}
0	3	1.77±0.02^{e}	0.79±0.04^{a}	2.24±0.08^{e}	6.83±0.12^{b}
	5	0.96±0.07^{ab}	0.78±0.14^{a}	1.24±0.31^{bcd}	4.27±0.23^{a}
	1	0.91±0.13^{ab}	0.90±0.20^{abc}	1.06±0.38^{abc}	8.95±0.27^{cd}
24	3	1.75±0.11^{e}	0.77±0.00^{a}	2.27±0.16^{e}	8.74±0.13^{cd}
	5	1.46±0.07^{cde}	0.81±0.07^{ab}	1.80±0.07^{de}	7.32±0.21^{b}
	1	0.77±0.12^{ab}	1.01±0.04^{abc}	0.77±0.34^{ab}	9.32±0.02^{def}
48	3	1.19±0.51^{bcd}	0.96±0.07^{abc}	1.22±0.44^{bcd}	9.61±0.32^{efg}
	5	0.78±0.11^{ab}	1.12±0.03^{bc}	0.70±0.12^{ab}	8.89±0.51^{cd}
	1	0.74±0.22^{ab}	0.87±0.05^{ab}	0.86±0.29^{ab}	10.17±0.16^{g}
72	3	0.99±0.11^{abc}	1.04±0.05^{abc}	0.96±0.16^{ab}	9.11±0.40^{cde}
	5	0.67±0.15^{a}	0.98±0.06^{abc}	0.67±0.21^{ab}	9.72±0.05^{fg}

All data were expressed as mean values±S.D. (n=6). Values followed by different letters in the same column are significantly different ($p<0.05$)

flasks at 0, 24, 48 and 72 h of fermentation. The results indicated that DCW and protein synthesis were inhibited by addition of methanol at the beginning of fermentation, which was similar to the above. As shown in Table 3, TGase production was improved in the culture supplemented with 1% and 3% of methanol at the beginning of fermentation. Addition of methanol after 48 h or later was not found to be beneficial for the production of TGase. However, 5% of methanol had a negative effect on TGase production. This might be because the higher methanol concentration in the medium disturbed *Streptomyces* metabolism and mycelia morphology, which resulted in a decreased TGase production. The maximum activity of TGase (1.77 and 1.75 U/ml) and TGase specific activity (2.24 and 2.27 U/mg protein) were obtained when 3% methanol was added to the medium at 0 and 24 h of fermentation.

3. Sodium Chloride Stress on TGase Production

Salt stress is an important factor which can activate the expression of a set of co-regulated 'general stress response' proteins such as SigB, SigN and SigH-like proteins and stimulate secondary metabolites in some bacteria [24-27]. Various concentrations of NaCl (0.05, 0.10, 0.15, 0.20 and 0.25 mol/*l*) were added into the basic medium at the beginning of fermentation of *S. mobaraensis*. The presence of salt in the medium led to a decrease in growth rate of *S. mobaraensis*, but the production of TGase was dramatically promoted (Fig. 1), indicating that disadvantageous condition for cell growth had a specific stimulative effect upon TGase production. TGase production was increased with the increasing concentration of salt from 0 to 0.2 mol/*l*. TGase production achieved the maximum of 3.0 U/ml at 0.20 mol/*l* NaCl and was 2.4 times as much as control (1.25 U/ml).

Effects of salt stress at different times of post inoculation were investigated by adding 0.20 mol/*l* NaCl into the fermentation liquid at 0, 12, 24, 48 and 72 h after inoculation, with the culture without NaCl as the control. TGase production and DCW of *S. mobaraensis* at 72 h of fermentation are shown in Fig. 2. Addition of salt during fermentation in the medium led to a decrease in growth rate of *S. mobaraensis*. In contrast, TGase production (1.2-3.0 U/ml) was significantly promoted compared with the control (1.1 U/ml). Cultures treated at 24, 48 and 72 h post inoculation failed to produce significant amounts of TGase production (1.2-1.8 U/ml) despite the fact that they had accumulated a higher biomass compared with the cultures with 0.2 mol/L salt at 0 h of fermentation. These results showed that optimum TGase production (3.0 U/ml) was obtained in the fermentation medium with 0.20 mol/*l* NaCl at the beginning of fermentation.

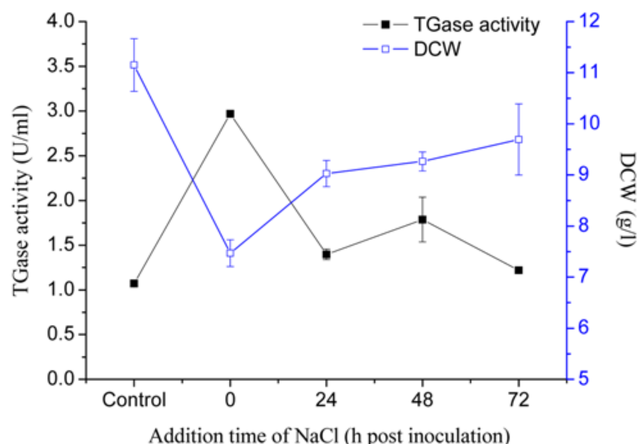

Fig. 2. Effects of addition periods for NaCl on TGase production and growth of *S. mobaraensis* DSM 40587. Culture without salt was the control. All data were expressed as mean values±S.D. (n=6).

4. Effects of Different Salts on the Production of TGase

To investigate effects of salts on TGase production, eight different salts including $MgCl_2$, NaCl, KCl, Na_2SO_4, $C_6H_5Na_3O_7$ (sodium citrate), Na_3PO_4, CH_3COONa and $CaCl_2$ were added into basic media at 0.2 mol/*l* and then autoclaved, with a basic medium serving as the control. Biomass and TGase level were determined and compared for all cultures at 96 h after being inoculated. Results showed that $CaCl_2$ could improve the biomass dry weight, but inhibited the formation of TGase significantly ($p<0.05$) (Fig. 3). The other salts inhibited strain growth in varying degrees. NaCl, KCl, Na_2SO_4, $C_6H_5Na_3O_7$, Na_3PO_4 and $MgCl_2$ could improve TGase production. It was noted

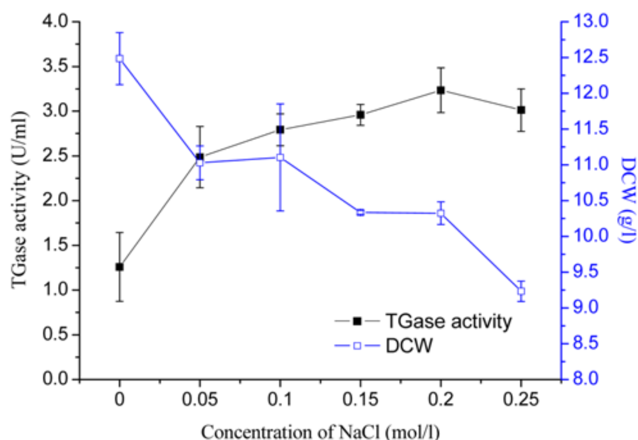

Fig. 1. Effects of NaCl concentration on TGase production and growth of *S. mobaraensis* DSM 40587 at the beginning of fermentation. All data were expressed as mean values±S.D. (n=6).

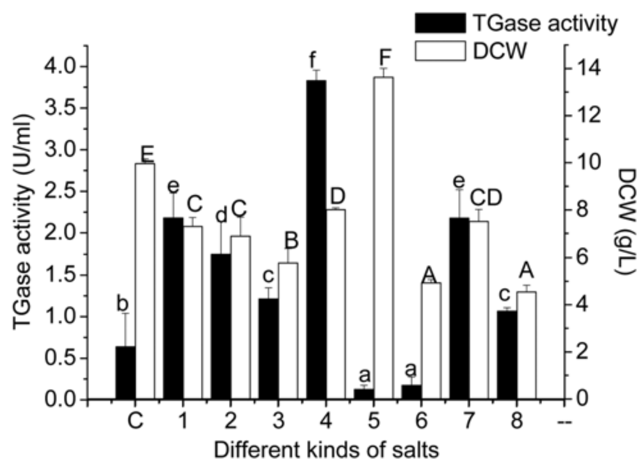

Fig. 3. Effects of salt kinds on the biomass and TGase activity at 96 h incubation. C-control, 1-NaCl, 2-Na_2SO_4, 3-$C_6H_5O_7Na_3$, 4-$MgCl_2$, 5-$CaCl_2$, 6-CH_3COONa, 7-KCl, 8-Na_3PO_4. All values are mean values±S.D. (n=6).

that $MgCl_2$ improved the enzyme production significantly, up to 3.8 U/ml, which was 3.5 fold higher than the control.

CONCLUSIONS

We found that TGase production was promoted by exposure to non-nutritional stresses, such as heat shock, supplementation of methanol and NaCl stress during fermentation. Optimal stress conditions for TGase production were the cultures treated at 60 °C after 48 h post inoculation, supplemented with 3% methanol or 0.2 mol/*l* $MgCl_2$ at the beginning of fermentation. Among these conditions, salt stress obtained the highest TGase production (3.8 U/ml), which was an increase in 3.5-fold compared with the control (1.25 U/ml). Further improvement of TGase by salt stress methods and their mechanism is currently being investigated intensively. In the method of enhanced production of TGase from *S. mobaraensis*, using salt stress might provide a significant impetus to improve economic feasibility and commercial viability of TGase for it was an economical and practical procedure to control TGase productivity on an industrial scale.

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