J Microbiol Immunol Infect
2001;34:8-16

Formation and regeneration of protoplasts for protease production in *Streptomyces rimosus*

Shang-Shyng Yang, Chiou-Mei Lei

Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan, ROC

Received: April 8, 2000 Revised: May 3, 2000 Accepted: May 21, 2000

To improve the formation and regeneration frequency of protoplasts for protease production, experiments were performed using a cultivation of *Streptomyces rimosus* TM-55 (CCRC 940061) in a Tryptic-soy broth (TSB) containing 2% of glycine for 2 days. It was found that the protoplast formation decreased with increased incubation temperature and increased ratio of culture broth to vessel volume. The optimal incubation temperature was 28 °C and the ratio of culture broth to vessel volume was 2:5. The hypertonic medium containing 10 mM $MgCl_2$, 25 mM $CaCl_2$ and 500 mM sucrose provided high stability for protoplasts. Supplementation with $MgSO_4$, KCl and $NaNO_3$ improved the regeneration frequency of protoplasts. The smear method had a higher protoplast regeneration frequency than the pour plate method. Protoplasts had protease productivity which was similar to that obtained with fresh mycelia, with each milliliter of culture broth yielding 141 units of protease with 3.5 x 10^8 protoplasts and 148 units of protease with 14.25 mg fresh mycelia respectively in a shaking culture, while the values were 15 and eight units of protease in a static culture.

Key words: Glycine, hypertonic medium, protease production, protoplast regeneration, *Streptomyces rimosus*

Members of the genus *Streptomyces* produce over 60% of all antibiotics, including more than 70% of all the commercial products [1]. Although proteases are mainly fungal and eubacterial products, the possibility of using streptomycetes for protease production has recently been investigated using organisms including *Streptomyces clavuligerus*, *Streptomyces erythreus*, *Streptomyces griseus*, *Streptomyces moderatus*, *Streptomyces rimosus* and *Streptomyces thermoviolaceus* [2-7]. However, the wild types of these mentioned species have low enzyme activities. Enzyme and antibiotic yields were shown to improve under optimized culture conditions and with strain improvement [8-11]. Recombination through conjugation is widespread in actinomycetes but this process has only been applied to a very limited extent to facilitate strain improvement due to the contact of the cell [1].

Protoplast fusion provides a new and promising method for studies in fundamental and applied genetics of metabolites production. Although preparation and regeneration of a high yield of protoplasts for metabolite production has been previously reported [12], the optimal incubation temperature and preparation conditions remain unclear. In this study, the effect of incubation temperature and metal ions on the preparation and the regeneration of protoplasts were investigated. The production of protease with protoplasts and fresh mycelia was also compared in a shaking and a static culture to assess the feasibility of protease production with protoplast.

Corresponding author: Professor Shang-Shyng Yang, Department of Agricultural Chemistry, National Taiwan University, No. 1, Section 4, Roosevelt Road, Taipei 10617, Taiwan, ROC.

Materials and Methods

Microorganism

S. rimosus TM-55 (CCRC 940061) was provided by Dr. Thomas H.H. Ku (Cyanamid Taiwan Corporation, Taiwan) for the formation of protoplasts and the production of oxytetracycline.

Culture media and conditions

Streptomyces was cultivated in an S-medium containing (g/L) glucose 10, yeast extract 4, Bacto peptone 4, $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 2, K_2HPO_4 4 and 20% glycine 100 mL at 28 °C for 1 day to the early stationary phase (absorbance was between 4 and 4.5 at 660 nm). Then the mycelia were transferred to a Tryptic-soy broth (TSB) (Merck, Germany) containing (g/L) Bacto dextrose 2.5, Bacto tryptone (pancreatic digest of casein) 17, Bacto soytone (pancreatic digest of soybean meal) 3, NaCl 5, K_2HPO_4 2.5 and 20% glycine 100 mL at 28 °C for 2 days. The protease production medium

was composed of (g/L) soluble starch 20, corn steep liquor (Sigma, USA) 10, $(NH_4)_2SO_4$ 6, $CaCO_3$ 8, NaCl 5 and soybean oil 2 at pH 7.5.

Yeast extract-malt extract medium (YEME-medium) containing (g/L) sucrose 340, glucose 10, yeast extract 3, malt extract 3, Bacto peptone 5 and 20% glycine 100 mL was used for mycelia growth. Yeast extract-dextrose medium (YD-medium) containing (g/L) yeast extract 10, glucose 10 and agar 18 at pH 7 was used for the seed culture and the nonprotoplast regeneration.

Hypertonic P-medium (P-medium) containing sucrose 103 g, $MgCl_2 \cdot H_2O$ 10.03 g, K_2SO_4 0.25 g, 0.5% KH_2PO_4 10 mL, 0.25 M TES buffer at pH 7.2 100 mL, 2.5 M $CaCl_2 \cdot H_2O$ 10 mL and trace element solution 4 mL in distilled water 870 mL (including $FeSO_4 \cdot 7H_2O$ 1 g, $CuSO_4 \cdot 5H_2O$ 0.5 g, $ZnSO_4 \cdot 7H_2O$ 1 g and $MnSO_4 \cdot 2H_2O$ 0.1 g/L), was used for protoplast preparation.

R2 medium containing (g/L) sucrose 171, glucose 10, gelatin 10, L-proline 3, $MgCl_2 \cdot 6H_2O$ 10.03, K_2SO_4 0.25, trace element solution 4 mL, 0.25 M TES buffer at pH 7.2 100 mL and 2.5 M $CaCl_2 \cdot 2H_2O$ 10 mL was used as the basal medium for protoplast regeneration [12].

Preparation of protoplasts

Mycelia were harvested by centrifugation (1000 x *g* for 30 min) (Kubota 2010, Japan) and washed twice with a P-medium. Then the mycelia were treated with 5 mL of 2 mg/mL lysozyme at 30 °C for 60 min to 120 min with occasional gentle shaking. Protoplasts were recovered by centrifugation at 500 x *g* for 20 min and then washed twice with a P-medium at 4 °C. The concentration of protoplasts was counted with a haemacytometer.

Regeneration of protoplasts

Protoplasts were resuspended in a P-medium and then smeared onto an regeneration medium (R-medium) or an YD medium at 28 °C for 7 to 10 days. The colonies formed on YD-medium were counted as the number of nonprotoplast mycelial fragments, while the colonies formed on R-medium were counted as the sum of protoplasts and nonprotoplast mycelial fragments. The regeneration frequency was calculated as follow:

$$\text{Regeneration frequency (\%)} = \frac{\text{Colonies on R-medium - Colonies on YD-medium}}{\text{Number of protoplasts used}}$$

Experiments were carried out to obtain three measurements, and statistical analysis of the results was performed using analysis of variance (ANOVA) and Duncan's multiple range test ($p = 0.05$) using statistical analysis system [13].

Sudan black B stain

Sudan black B stain solution was prepared as follows: Sudan black B 0.3 g dissolved in 100 mL ethanol was heated to 40 °C and filtered with Whatman No. 1 filter paper. Protoplast suspension 1 mL and Sudan black B 0.2 mL were reacted at 40 °C for 30 min to 60 min, and centrifugated at 3500 x *g* for 5 min. The supernatant was diluted with distilled water and the absorbance at 588 nm was measured. The cell precipitates were observed under a light microscope (Olympus BH-2, Japan). The protoplast was stained with Sudan black B and became blue black to black in color, while the conidium remained transparent on application of Sudan black B.

Protease activity

Approximately 3.8×10^8 protoplasts or fresh mycelia 14.25 mg was inoculated in 100 mL of protease production broth and statically cultivated at 28 °C for 10 days. A second set of broth was cultivated at 28 °C and 150 rev/min shaking culture for 10 days. After cultivation, protease activity in the culture broth was determined using a modification of Anson's method [10]. The reaction mixtures containing 1 mL of 0.6% casein in 0.2 M phosphate buffer at pH 7 and 1 mL of enzyme solution were incubated at 37 °C for 20 min, and the reaction was stopped with 3 mL of 10% trichloroacetic acid. The absorbance of liberated tyrosine in the filtrate was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme that produced an absorbance at 280 nm equivalent to 1 μmol of tyrosine in 1 min under the assay conditions.

Results

Effects of metal ions and sucrose on protoplast stability

The effect of calcium and magnesium ions on the stability of protoplasts is listed in Table 1. The presence of calcium and magnesium ions in a P-hypertonic solution improved the stability of protoplasts. The stability of protoplasts significantly increased with the supplement of 10 mM to 25 mM $CaCl_2$ and $MgCl_2$ ($p < 0.05$). This effect decreased when the concentration of calcium or magnesium ions was higher than 25 mM. In addition, the P-medium containing 300 mM sucrose also increased the stability of protoplasts. The regeneration of protoplasts was enhanced with sucrose supplement

Table 1. Effects of calcium ion, magnesium ion and sucrose concentration on the absorbance and leakage of protoplasts

$CaCl_2$ (mM)	$MgCl_2$ (mM)	Sucrose (mM)	Absorbance (A_{260nm})	Leakage (%)
0	0	0	1.105 ±0.034	100.0^{a}
0	0	300	0.810 ±0.062	73.3^{b}
10	10	300	0.578 ±0.022	52.3^{c}
10	25	300	0.371 ±0.008	33.6^{e}
25	10	300	0.440 ±0.030	$39.8d^{e}$
25	25	300	0.405 ±0.016	36.7^{e}
25	50	300	0.525 ±0.042	47.5^{cd}
50	25	300	0.408 ±0.034	36.9^{e}
50	50	300	0.517 ±0.036	46.8^{cd}

Basal medium contained K_2SO_4 0.25 g, 0.5% KH_2PO_4 10 mL, trace element solution 4 mL, 0.25 M TES buffer 100 mL at pH 7.2 and distilled water 876 mL. Mean ±SD (n = 3). Means in the same column which do not share the same alphabetic superscript (*a*, *b*, *c*, *cd*, *d*, and e) are significantly different at a 5% level according to the results of Duncan's multiple range test. Absorbance of the control medium without $CaCl_2$, $MgCl_2$ and sucrose at 260 nm was considered as 100% leakage.

Table 2. Effects of sucrose and lysozyme concentration on protoplast formation

Sucrose (mM)	Lysozyme (mg/mL)	Reaction period (h)	Protoplast (x 10^6/mL)
300	1	1.5	34.0 ± 11.5^{b}
300	2	1.5	108.0 ± 23.0^{a}
300	2	2.0	122.0 ± 14.4^{a}
500	1	3.5	3.2 ± 0.7^{d}
500	2	3.0	33.6 ± 3.7^{b}
500	2	3.5	35.0 ± 2.3^{b}

Basal medium was P-medium without sucrose. Mean ±SD (n = 3). Means in the same column which do not share the same alphabetic superscript (*a*, *b* and *d*) are significantly different at the 5% level according to the results of Duncan's multiple range test.

and reached a maximum value at 500 mM of sucrose (data not shown). However, the size and the stability of protoplasts decreased at 500 mM sucrose. Therefore, the concentration of sucrose used in the P-medium for protoplast formation was adjusted to 300 mM sucrose. Optimal protoplast formation was obtained by treatment of mycelium with 2 mg/mL lysozyme dissolved in a P-medium containing 300 mM sucrose at 30 °C for 1.5 h to 2 h. Each milliliter produced 1.08 (±0.23) to 1.22 (±0.14) x 10^8 protoplasts. Increasing the concentration of sucrose or the duration of the reaction period significantly reduced the protoplast formation (Table 2).

Effects of medium composition on protoplast regeneration

Protoplast regeneration increased with the addition of casamino acid or gelatin and attained the maximum value at 0.5% of casamino acid (regeneration was 4 ±1.24%) or 1% of gelatin (regeneration was 10 ±1.92%). The effect of medium composition on protoplast regeneration is shown in Table 3. Protoplast regeneration increased from 0.41 ±0.2% in R2 medium, to 3.04 ±1.42 % in RY medium, and to 4.31 ±1.8% in R6 medium with the supplement of yeast extract and sucrose. The yields were significantly different among RY-, R6- and R2-media ($p < 0.05$). The protoplast regeneration ranged from 10.32 ±3.21 to 13.26 ±4.62% in the R6 series of media with the supplementation of casamino acid, $MgSO_4$ $7H_2O$, KCl and $NaNO_3$. Significant to very significant differences in protoplast

Table 3. Effects of media composition on protoplast regeneration

Medium	Sucrose (mM)	Yeast extract (g/L)	Casamino acid (g/L)	$MgCl_2$ (mM)	$MgSO_4$ $7H_2O$ (x 10^{-3} mM)	KCl (x 10^{-2} mM)	$NaNO_3$ (x 10^{-2}mM)	Regeneration (%)
R2	500	0.0	0.0	50	0.0	0.0	0.0	0.41 ± 0.21^{c}
RY	500	10.0	0.0	50	0.0	0.0	0.0	3.04 ± 1.42^{bc}
R6	600	10.0	0.0	50	0.0	0.0	0.0	4.31 ± 1.82^{bc}
R6-0	600	10.0	0.0	50	1.66	1.83	2.35	12.42 ± 1.63^{a}
R6-1	600	10.0	0.4	50	1.66	1.83	2.35	13.26 ± 4.62^{a}
R6-2	600	10.0	0.4	20	1.66	1.83	2.35	10.32 ± 3.22^{a}

Mean ±SD (n = 3). Means in the same column which do not share the same alphabetic superscript (*a*, *bc* and *c*) are significantly different at the 5% level according to the results of Duncan's multiple range test. The initial protoplast concentration was 4 x10^7/mL.

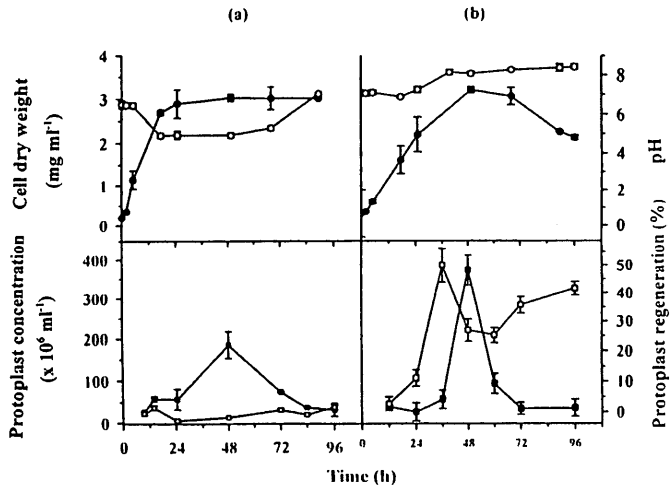

Fig. 1. Time course of protoplast formation and regeneration of mycelia grown in S- and TSB-media.
(a) S-medium. (b) TSB-medium.
O–O pH
●–● Cell dry weigh
❑–❑ Protoplast concentration
■–■ Protoplast regeneration

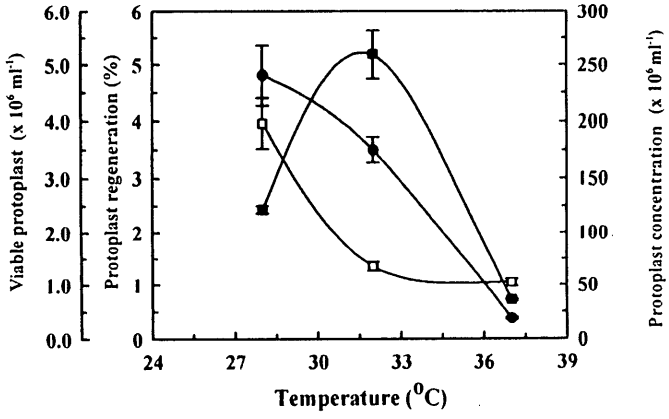

Fig. 2. Effects of incubation temperature on protoplast formation and regeneration.
❑–❑ Protoplast concentration
■–■ Protoplast regeneration
●–● Viable protoplasts

regeneration were found between R6- and RY-media ($p < 0.05$) or between R6- and R2-media ($p < 0.01$). The media R6-0 and R6-1 were found to be most suitable for *Streptomyces* protoplast regeneration.

The effect of agar concentration on protoplast regeneration is shown in Table 4. Protoplast regeneration was reduced with increasing agar concentration in the regeneration medium by both the smear method and the pour plate method. The optimal concentration of agar for protoplast regeneration was between 1% and 1.2% with the smear method (the medium could not solidify when the agar concentration was less than 1%) and between 0.7% and 1.2% with the pour plate method. The colony morphology changed with the concentration of agar. The colony appeared smooth with the smear method in the regeneration medium containing 1% to 1.2% agar, but became shrunk and rough when the agar concentration was greater than 1.5%. The mycelial fragment was thin at 2% agar and thick at 0.7% to 1.2% agar with the pour plate method.

Table 4. Effects of agar concentration and plating method on protoplast regeneration

Agar (%)	Smear method (%)	Pour plate method (%)
0.7	-	8.3 ±1.9^{c}
1.0	28.5 ±5.5^{a}	5.6 ±0.8^{c}
1.2	25.1 ±3.1^{a}	4.5 ±0.4^{c}
1.5	18.6 ±2.1^{b}	2.5 ±0.5^{c}
1.8	14.8 ±0.9^{bc}	1.4 ±0.3^{d}
2.0	11.2 ±0.8^{bc}	0.7 ±0.1^{d}

R6-1 regeneration medium without agar was used as the basal medium. The initial protoplast concentration was 2 x 10^{7} mL^{-1}. Mean ±SD (n = 3). Means in the same column which do not share the same alphabetic superscript (*a*, *b*, *bc*, *c* and *d*) are significantly different at the 5% level according to the results of Duncan's multiple range test.

Protoplast regeneration frequency with the smear method was higher than the pour plate method at the same concentration of agar. There was a significant difference between these two methods in the protoplast regeneration frequency at 1% agar, with 28.5 ±5.4% for the smear method and 5.6 ±0.8% for the pour plate method.

Autoinhibition of protoplasts

When the protoplast suspensions were spread on regeneration medium at 28 °C for 5 days, colonies grown from mycelium fragments were of large size and quick growing, whereas those regenerated from protoplasts were of small size and slow growing. There was no autoinhibition phenomenon between colonies regenerated from mycelium fragments and protoplasts, or between protoplasts or mycelium fragments themselves.

Effects of environmental conditions on protoplast formation

Cultivation period and medium

Streptomyces cultivated in both S-medium and TSB-medium entered the log phase before 24 h, stationary phase between 24 h and 96 h, and declining phase after 96 h incubation. The pH value dropped from 6.6 to 5 in the log phase, from 5 to 5.2 in the stationary phase, and then increased to 8.3 in the declining phase. Protoplast formation peaked in the late log phase to the late stationary phase. Each milliliter produced 4 (±0.5) x 10^{7} and 3.9 (±0.4) x 10^{8} protoplasts in S-medium and TSB-medium, respectively. The protoplast preparation with mycelium growth in the middle stationary phase produced the maximal regeneration frequency, with 22% and 50% for S-medium and TSB-medium, respectively (Fig. 1). Protoplast formation and regeneration from TSB-medium was found to be higher than that from S-medium ($p < 0.05$). However, the diameter of protoplasts from TSB-medium ranged from 1.8 μm to 2.3 μm while those prepared from S-medium ranged from 2 μm to 2.5 μm.

Incubation temperature

The effect of incubation temperature of mycelium on protoplast formation and regeneration is shown in Fig. 2. The results indicate that the optimal incubation temperature of mycelium for protoplast formation was 28 °C and for regeneration was 32 °C. The protoplast

Table 5. Effects of medium, glycine concentration and lysozyme reaction time on protoplast formation and regeneration

Medium	Glycine (%)	Lysozyme reaction period (h)	Protoplast concentration (x 10^{6}/mL)	Regeneration (%)	Total viable protoplasts (x 10^{6}/mL)
S	0	2.0	1.0 ±0.2^{d}	-	-
	1	2.0	5.0 ±1.0^{d}	20.0 ±6.2^{b}	1.0 ±0.4^{c}
	2	1.0	62.1 ±5.2^{c}	21.2 ±4.8^{b}	13.1 ±3.0^{b}
	2	2.0	122.1 ±10.1^{b}	3.6 ±1.0^{c}	4.4 ±1.2^{c}
	3	2.0	133.3 ±11.2^{b}	3.1 ±1.6^{c}	4.2 ±1.6^{c}
	4	2.0	190.2 ±18.4^{a}	2.9 ±0.8^{c}	5.5 ±1.5^{c}
TSB	0	1.5	12.1 ±3.2^{cd}	-	-
	1	1.5	104.2 ±18.2^{b}	45.8 ±9.6^{a}	47.6 ±9.3^{ab}
	2	1.5	173.3 ±13.3^{a}	51.2 ±10.1^{a}	88.6 ±18.2^{a}
	3	1.5	216.1 ±15.2^{a}	30.8 ±6.6^{ab}	66.5 ±10.6^{a}
YEME	0	2.5	0.5 ±0.1^{d}	-	-
	1	2.5	2.1 ±0.7^{d}	62.1 ±8.8^{a}	1.2 ±0.4^{c}
	2	2.5	21.2 ±5.2^{cd}	52.6 ±9.3^{a}	11.0 ±3.8^{b}
	3	2.5	32.4 ±4.1^{c}	49.4 ±7.6^{a}	15.8 ±4.9^{b}

Abbreviations: S = S-medium; TSB = Tryptic-soy broth; YEME = yeast extract-malt extract

Mean ±SD (n = 3). Means in the same column which do not share the same alphabetic superscript (*a*, *ab*, *b*, *c*, *cd* and *d*) are significantly different at the 5% level according to the results of Duncan's multiple range test.

Table 6. Effects of volume ratio on protoplast formation and regeneration

Medium	Volume ratio	Lysozyme reaction time (h)	Protoplast		
			Conc. (x 10^6/mL)	Regeneration (%)	Viable count (x 10^6/mL)
S	1:5	2.0	134 ±22^{bc}	0.0 ±0.0^{b}	0.0 ±0.0^{b}
	2:5	2.0	122 ±14^{bc}	3.6 ±1.0^{b}	4.4 ±1.2^{b}
	3:5	2.0	104 ±16^{c}	1.2 ±0.4^{b}	1.2 ±0.4^{b}
TSB	1:5	1.5	220 ±35^{a}	8.8 ±2.1^{b}	19.5 ±4.6^{b}
	2:5	1.5	173 ±23^{bc}	51.2 ±22.1^{a}	125.1 ±38.2^{a}
	3:5	1.5	142 ±14^{bc}	18.6 ±3.6^{ab}	26.4 ±5.1^{b}
YEME	1:5	2.5	83 ±11^{c}	29.3 ±6.3^{ab}	24.3 ±5.2^{b}
	2:5	2.5	21 ±5^{cd}	52.6 ±18.3^{a}	11.0 ±3.8^{b}
	3:5	2.5	14 ±6^{d}	23.4 ±5.4^{ab}	3.3 ±0.8^{b}

Abbreviations: S = S-medium; TSB = Tryptic-soy broth; YEME = yeast extract-malt extract
Mean ±SD (n = 3). Means in the same column which do not share the same alphabetic superscript (*a*, *ab*, *b*, *bc*, *c* and *d*) are significantly different at the 5% level according to the results of Duncan's multiple range test.

Table 7. Effects of storage temperature and duration on protoplast regeneration

Protoplast concentration (number/mL)	Storage temperature (°C)	Storage duration (days)				
		0	0.5	1.0	2.0	4.0
		----------	----------	----------	----------	----------
652,000	5	> 6.13^{a}	> 4.62^{a}	> 3.07^{a}	> 2.30^{a}	0.94 ±0.11
	10	> 6.13^{a}	> 4.60^{a}	>2.87^{a}	0.06 ±0.01	0.06 ±0.01
	15	> 6.13^{a}	> 3.72^{a}	> 2.29^{a}	0.06 ±0.01	0.05 ±0.01
725,000	20	> 5.89^{a}	> 2.43^{a}	0.56 ±0.03	0.06 ±0.01	0.05 ±0.01
	25	> 5.89^{a}	> 1.63^{a}	0.59 ±0.04	0.02 ±0.01	< 0.01^{b}
	30	> 5.89^{a}	0.47 ±0.02	0.22 ±0.01	0.11 ±0.01	< 0.01^{b}
65,200	5	4.98 ±0.14	3.29 ±0.21	2.74 ±0.14	1.03 ±0.02	0.25 ±0.01
	10	4.98 ±0.14	3.86 ±0.14	1.64 ±0.02	< 0.01^{b}	< 0.01^{b}
	15	4.98 ±0.14	3.11 ±0.12	1.64 ±0.04	< 0.01^{b}	< 0.01^{b}
72,500	20	1.61 ±0.05	1.74 ±0.07	< 0.01^{b}	< 0.01^{b}	< 0.1^{b}
	25	1.61 ±0.05	1.13 ±0.05	< 0.01^{b}	< 0.01^{b}	< 0.1^{b}
	30	1.61 ±0.05	0.07 ±0.01	< 0.01^{b}	< 0.01^{b}	< 0.1^{b}
6,520	5	3.94 ±0.18	0.74 ±0.06	0.36 ±0.13	0.05 ±0.01	0.03 ±0.01
	10	3.94 ±0.18	0.56 ±0.04	0.20 ±0.04	< 0.01^{b}	< 0.01^{b}
	15	3.94 ±0.18	< 0.01^{b}	< 0.01^{b}	< 0.01^{b}	< 0.01^{b}
7,250	20	6.14 ±0.34	< 0.01^{b}	< 0.01^{b}	< 0.01^{b}	< 0.01^{b}
	25	6.14 ±0.34	< 0.01^{b}	< 0.01^{b}	< 0.01^{b}	< 0.01^{b}
	30	6.14 ±0.34	< 0.01^{b}	< 0.01^{b}	< 0.01^{b}	< 0.01^{b}

S-medium was used for storage of the protoplasts. R6-1 medium was used for the regeneration of protoplasts. Mean ±SD (n = 3).
[a]The colony numbers were more than 1000 on the plate.
[b]The colony numbers were less than 10 on the plate.

regeneration frequency was lower at 28 °C than that at 32 °C, whereas the total number of viable protoplasts was higher at 28 °C than that at 32 °C. Therefore, mycelia were cultivated at 28 °C for protoplast preparation and regeneration.

Medium composition

The effect of medium composition on protoplast formation and regeneration is shown in Table 5. Supplementation with glycine significantly enhanced the protoplast concentration in all culture media. The protoplast regeneration frequency was significantly decreased in S-medium compared to TSB- and YEME-media. The total number of viable protoplasts was not affected by the addition of glycine. The optimal glycine concentration for protoplast formation and regeneration was 2%. Each milliliter produced 6.2 (±1.5) x 10^7, 1.73 (±0.23) x 10^8, and 2.1 (±1.5) x 10^7 protoplasts for S-, TSB-, and YEME-media, respectively. The regeneration frequency was 21.2 ±4.8%, 51.2 ±22.1%, and 52.6 ±18.3%, respectively.

Volume ratio

At 150 rev/min shaking culture, the optimal volume

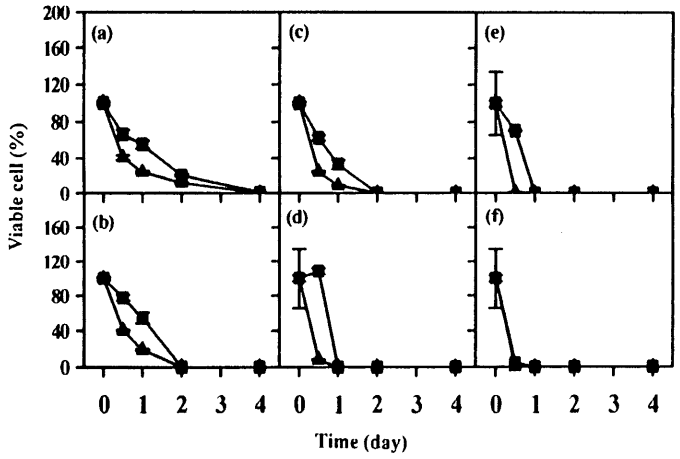

Fig. 3. Effects of storage temperature on protoplast formation and regeneration.
(a) 5 °C (b) 10 °C (c) 15 °C (d) 20 °C (e) 25 °C (f) 30 °C
▲-▲ Protoplasts
■-■ Nonprotoplast fragments

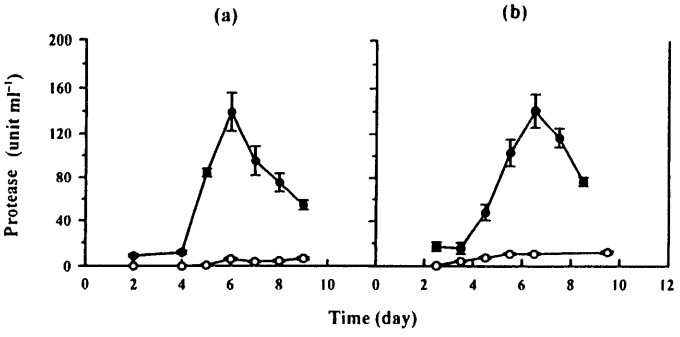

Fig. 4. Effects of aeration on protease production with protoplast and fresh mycelia.
(a) Fresh mycelia (b) Protoplasts
●-● Shaking culture
○-○ Static culture

ratio of culture broth to vessel volume in mycelia cultivation for protoplast formation and regeneration was 2:5 in all tested media. Protoplast formation gradually decreased with the increase of the volume ratio from 1:5 to 3:5, and the regeneration had the highest value at 2:5. The total number of viable protoplasts also had a high value at the volume ratio of 2:5. The use of a low volume ratio increased the dissolving of oxygen and enhanced protoplast formation. Each milliliter of broth produced 1.22 (±0.14) x 10^8, 1.73 (±0.23) x 10^8, and 2.10 (±0.5) x 10^7 protoplasts at volume ratio 2:5 for S-, TSB-, and YEME-media, respectively (Table 6).

Storage temperature and duration

The effect of storage temperature and duration on the regeneration of protoplasts is shown in Table 7 and Fig. 3. Low temperature and high concentration of protoplasts favored protoplast storage and regeneration. Protoplast regeneration decreased with increased storage duration and temperature. However, a high concentration of protoplasts increased the regeneration frequency. In addition, protoplasts were more stable than nonprotoplast mycelia fragments during the storage. The percentage of total viable cells differed significantly between protoplasts and nonprotoplast mycelia fragments during storage at 5 °C to 25 °C, but these differences were insignificant for storage at 30 °C.

Protease production with protoplasts and mycelia

The effect of the various culture methods on protease

production with protoplasts and fresh mycelia is shown in Fig. 4. Protease activities increased with the cultivation of 3.5×10^8 protoplasts or 14.25 mg fresh mycelia in a shaking culture, and reached peak levels on the sixth and seventh day. Each milliliter of broth produced 141 and 148 units of protease with protoplasts and with fresh mycelia in shaking cultures, respectively, while only 15 and 8 units of protease were produced in the respective static cultures. High aeration favored protease production in both protoplast and fresh mycelia. Protoplast and fresh mycelia yielded similar amounts of protease using both culture methods.

Discussion

Microbial protoplast preparation is usually performed by mechanical removal of cell wall, and enzymatic digestion of cell wall with helix pomatia or lysozyme. Several methods have been designed in an attempt to improve protoplast preparation and regeneration in *Streptomyces*, including glycine treatment of mycelia, use of a mixture of lysozyme and lytic enzyme, and supplementation of metal ions and osmotic stabilizers [12,14,15]. In this study, it was shown that 10 mM to 25 mM of calcium and magnesium ions and 300 mM sucrose could stabilize the protoplasts and prevent the leakage of protoplasts. Reaveley and Rogers [16] reported that magnesium ion could prevent the release of lipid residue in the plasma membrane and mesosome from the protoplasts of *Bacillus*. Pigac *et al* [15] reported that calcium, magnesium and sucrose could stabilize protoplasts, thereby preventing leakage. However, in their study, 1000 mM of sucrose reduced the formation and the stability of protoplasts.

Supplementation of casamino acids and gelatin increased the regeneration frequency of protoplasts. Miller *et al* [17] found that 25% gelatin stimulated the growth of L-form *Bacillus*. Gabor and Hotchkiss [18] also reported that amino acids and proteins had a stimulating effect on protoplast regeneration. In addition, some researchers indicated that partial dehydration of regeneration medium, low temperature and modification in plating methods (such as overlay a soft agar) could improve protoplast regeneration [15, 19]. In this study, we found that the use of 1% to 1.2% agar in the smear method and 0.7% to 1.2% agar in the pour plate method favored protoplast regeneration. The smear method had a significantly higher regeneration rate than the pour plate method. This might have been due to the thermal sensitivity of the protoplasts of *S. rimosus*. However, Baltz and Matsushima [19] and Pigac *et al* [15] reported that 0.3% to 0.65% soft agar overlaid on the regeneration media significantly enhanced the protoplast regeneration of *Streptomyces*.

Hopwood and Merrick [1] reported that the protoplast regeneration in *Streptomyces acrimycini* and *Streptomyces fradiae* had an autoinhibitory effect in the presence of other protoplasts or nonprotoplast mycelia fragments. This phenomenon had not been reported in this study, but it was shown that a high concentration of protoplasts improved the stability during storage. A similar result was also demonstrated in *S. rimosus* by Pigac *et al* [15].

In the present study, protoplast formation was high in the early stationary phase and decreased in the late stationary phase. This phenomenon is consistent with the results of Hopwood and Merrick in *S. acrimycini* and *S. fradiae* [1]. In addition, protoplast formation also increased in the decline phase, likely due to the autolysis of mycelia and the partial damage of the cell wall in this stage. Rodicio *et al* [20] found that protoplasts were yielded in young mycelia treated with lysozyme, even when glycine was not added during the growth period; however, protoplast formation in old mycelia was required in the presence of glycine during the cultivation. Therefore, mycelium grown at the early to the middle log phase treated with lysozyme were effective when used for protoplast preparation.

Glycine has been used to replace D-alanine in cell wall peptidoglycans and thereby enhance the effectiveness of lysozyme in lysing the *Streptomyces* cell wall for protoplast formation [21]. The addition of glycine caused some growth retardation and mycelium was much more sensitive to lysozyme in *Streptomyces* [20]. A high concentration of glycine or a prolonged treatment period inhibited the incorporation of D-alanine into the cell wall and reduced the regeneration frequency of protoplasts [12,22].

Protoplasts were more sensitive to high temperature (> 30 °C) than mycelium. Therefore, the storage the protoplasts at low temperatures (around 5 °C) stabilizes protoplast activity. Eisenberg and Corner [23] found that protoplasts of *Bacillus megaterium* had different osmotic pressure-tolerances when they were cultivated at different temperatures because of differences in the fatty acid composition of the cell membrane.

Protoplast regeneration frequency is estimated by the ratio of cell number to protoplast number. Baltz and Matsushima reported that cell regeneration from the protoplasts of *Streptomyces* depends on the medium components, age of mycelia, dehydration of the plates, and culture temperature [19]. In this study, it was also found that the presence of nonprotoplast mycelium fragments could affect the cellular regeneration from protoplasts. Glycine addition could enhance protoplast

formation but reduce the cell regeneration from protoplasts.

In the present study, shaking treatment stimulated the production of protease. Zhou *et al* [24] and Andres *et al* [25] reported that aeration improved the production of 7,10-dihydroxy-8(E)-octadecanoic acid and cephalosporin C. A low level of dissolved oxygen inhibited the microbial growth, antibiotic production and lipid consumption [26]. Chen and Wilde found that protoplast regeneration changed cell physiology and affected the production of metabolites. Keller [27] reported that the protoplasts of *Claviceps purpurea* produced less alkaloid than the whole cell. In contrast, Ikeda *et al* [28] and Malina *et al* [29] reported that the protoplasts of *S. fradiae*, *Streptomyces ambofaciens*, *Streptomyces cirratus* and *Streptomyces incarnatus* had higher antibiotic productivity than mycelia. In this study, the protoplasts and fresh mycelia of *S. rimosus* yielded the similar protease activities. Use of protoplasts for the production of primary and secondary metabolites has important potential as a future technology for the production of antibiotics.

References

1. Hopwood DA, Merrick MJ. Genetics of antibiotic production. Bacteriol Rev 1977;41:595-635.
2. Chandrasekaran S, Dhar SC. Multiple proteases from *Streptomyces moderatus*. I. Isolation and purification of five extracellular proteases. Arch Biochem Biophys 1987;257:395-401.
3. Bascaran V, Hardisson V, Brana A. Regulation of extracellular protease production in *Streptomyces clavuligerus*. Appl Microbiol Biotechnol 1990;34:208-3.
4. James PDA, Iqbal M, Edwards C, Miller PGG. Extracellular protease activity in antibiotic-producing *Streptomyces thermoviolaceus*. Curr Microbiol 1991;22:377-82.
5. Muro T, Murakami T, Tominaga Y, Tokuyama T, Okada S. Purification and some properties of protease I having transfer action from *Streptomyces griseus* var. *alcalophilus*. Agric Biol Chem 1991;55:307-14.
6. Yeoman KH, Edwards C. Protease production by *Streptomyces erythreus*. FEBS Lett 1994;15:129-32.
7. Yang SS, Wang JY. Protease and amylase production of *Streptomyces* in submerged and solid state cultivations. Bot Bull Acad Sin 1999;40:259-65.
8. Hranueli D, Pigac J, Smokvina T, Alacevic M. Genetic interactions in *Streptomyces rimosus* mediated by conjugation and by protoplast fusion. J Gen Microbiol 1983;129:1415-22.
9. Yang SS, Ling MY. Tetracycline production with sweet potato residue by solid state fermentation. Biotechnol Bioeng 1989; 33:1021-8.
10. Yang SS, Huang CI. Protease production by amylolytic fungi in solid state fermentation. Chin J Agric Chem Soc 1994;32: 589-601.
11. Cheng CW, Lin CS, Liu YT, Yang SS. Cloning and expression of the α-amylase gene and oxytetracycline production in *Streptomyces rimosus*. World J Microbiol Biotechnol 2000;16: 225-30.
12. Yang SS, Kao CY. Oxytetracycline production in solid state and submerged fermentation by protoplast fusants of *Streptomyces rimosus*. Proc Natl Sci Counc ROC 1991;15:20-7.
13. SAS Institute. SAS/STAT User's Guide. Release 6.03 SAS Institute, North Carolina: Cary, 1988.
14. Ogawa H, Imai S, Satoch A, Kojima M. An improved method for the preparation of *Streptomycetes* and *Micromonospora protoplasts*. J Antibiot 1983;36:184-6.
15. Pigac J, Hranueli D, Smokvina T, Alacevic M. Optimal cultural and physiological conditions for handling *Streptomyces rimosus* protoplasts. Appl Environ Microbiol 1982;44:1178-86.
16. Reaveley DA, Rogers HJ. Some enzymatic activities and chemical properties of the mesosomes and cytoplasmic membranes of *Bacillus licheniformis* 6346. Biochem J 1969; 113:67-79.
17. Miller IL, Weibe W, Landman OE. Gelatin-induced reversion of protoplasts of *Bacillus subtilis* to the bacillary form: photomicrographic study. J Bacteriol 1968;96:2171-4.
18. Gabor MH, Hotchkiss RD. Parameters governing bacterial regeneration and genetic recombination after fusion of *Bacillus subtilis* protoplasts. J Bacteriol 1979;137:1346-53.
19. Baltz RH, Matsushima P. Protoplast fusion in *Streptomyces*: conditions for efficient genetic recombination and cell regeneration. J Gen Microbiol 1981;127:137-46.
20. Rodicio MR, Manzanal MB, Hardisson C. Protoplast formation during spore germination in *Streptomyces*. Curr Microbiol 1978; 1:89-92.
21. Sagara Y, Fukui K, Ota F, Yoshida N, Kashiyama T. Rapid formation of protoplasts of *Streptomyces griseoflavus* and their fine structure. Jpn J Microbiol 1971;15:73-84.
22. Scheu K, Soliveri J, Arias E. Factors affecting protoplasts formations and regeneration in three strains of *Streptoverticillium*. J Microbiol Methods 1994;19:197-206.
23. Eisenberg AD, Corner TR. Osmotic behavior of bacterial protoplasts: temperature effects. J Bacteriol 1973;114:1177-83.
24. Zhou W, Holzhauer-Rieger K, Dors M, Schugerl K. Influence of dissolved oxygen concentration on the biosynthesis of cephalosporin C. Enzyme Microb Technol 1992;14:848-54.
25. Andres CD, Mercade E, Guinea J, Manresa A. 7,10-Dihydroxy-8(E)-octadecanoic acid produced by *Pseudomonas* 42A2: evaluation of different cultural parameters of the fermentation. World J Microbiol Biotechnol 1994;10:106-9.
26. Chen HC, Wilde F. The effect of dissolved oxygen and aeration rate on antibiotic production of *Streptomyces fradiae*. Biotechnol Bioeng 1991;37:591-5.
27. Keller U. Highly efficient mutagenesis of *Claviceps purpurea* by using protoplasts. Appl Environ Microbiol 1983;46:580-4.
28. Ikeda H, Tanaka H, Omura S. Improvement of macrolide antibiotic producing *Streptomyces* by regeneration of protoplast. J Antibiot 1983;36:283-8.
29. Malina H, Tempete C, Robert-Gero M. Enhanced sinefungin production by medium improvement, mutagenesis, and protoplast regeneration of *Streptomyces incarnatus* NRRL 8089. J Antibiot 1985;38:1204-10.