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Conditions for Intergeneric Protoplast Fusion of Yeast

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酵母의 異屬間 原形質体 融合條件

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Optimum conditions of PEG treatment for the intergeneric fusion of yeast protoplasts were investigated. Fusants were selected by nutritional complementation on minimal medium. The intergeneric fusion frequency between protoplasts of *S. cerevisiae* and *C. tropicalis* was distributed 10^{-4} to 10^{-6}, depending on the combination of parental strains. PEG 4000 or 6000 are equally effective. 30 %(w/v) PEG 4000 was found to be optimum and below 20 % its stabilizing effect was lost, resulting in protoplast lysis, and optimum pH was 8.0. The efficiency of PEG was enhanced by higher temperature of the PEG solution, and by the addition of Ca ions. The stimulating effect of Ca ions in the range of 1 mM to 100 mM proved similar.

Since the fusogenic property of polyethylene glycol (PEG) to plant protoplasts was discovered,[1] this polyol has been used for protoplast fusion of many different organisms including animal cells,[2,3] fungi,[4,5,6,7] bactena,[8,9,10,11] and yeasts.[12,13,14]

The first successful fusion of yeast protoplast with PEG was performed with auxotrophic strains of *Schizosaccharomyces pombe*[12] and a number of investigations have been reported demonstrating the use of protoplast fusion in the breeding of yeasts.[15,16,17,18,19,20,21] However, in practice there are few examples of stable hybrid formation when distantly related species are fused.

In this study, we describe the influence of different conditions of PEG treatment on the intergeneric fusion of yeast protoplasts in an attempt to introduce starch utilization into *S. cerevisiae*. The protoplast fusion was detected by nutritional complementation between auxotrophic mutants of parental strains.

Materials and Methods

Strains

Auxotrophic mutants of *S. cerevisiae* HAKKOKEN 1 GO and *C. tropicalis* IFO 0589 were selected after treatment with N-methyl-N'-nitro-N-nitrosoguanidine[22] and are listed in Table 1. The successive mutagenic treatment with U.V. gave diauxotrophic mutants.

The procedure to induce auxotrophs involved an enrichment process with an antibiotics, Amphotericin B, according to the method of Most.[23] These auxotrophic gene markers were stable over several vegetative generations and strains were maintained on YPD medium.

Media

The yeasts for protoplast fusion were cultured aerobically, with shaking, to exponential phase in production medium.[24]

The YPD agar medium containing 20g of dextrose, 5g of

Table 1. List of strains used.

Strains		Phenotype	Origin
S. cerevisiae	HAKKOKEN 1 GO	Wild type	
	RSC-60	Folic$^-$, Panto$^-$	Mutants induced in *S. cerevisiae* HAKKOKEN 1 GO.
	RSC-86	Panto$^-$	
	RSC-87	Ile$^-$	
	RSC-91	Trp$^-$	
	RSC-111*	Lys$^-$, Glu$^-$	
	RSC-113*	Lys$^-$, Val$^-$	
	RSC-131	Arg$^-$, Orn$^-$	
	RSC-139	Thr$^-$, Cys$^-$	
	RSC-216	Lys$^-$	
C. tropicalis	IFO 0589	Wild type	
	RCT-40	Lys$^-$	Mutants induced in *C. tropicalis* IFO 0589.
	RCT-67	Val$^-$	
	RCT-82	Phe$^-$, Arg$^-$	
	RCT-84	Ser$^-$, Trp$^-$	
	RCT-104	Ala$^-$,	
	RCT-144**	Lys$^-$, Leu$^-$	
	RCT-167	Leu$^-$, His$^-$	

* uv mutant of RSC 216, ** uv mutant of RCT 40.

peptone, 5g of yeast extract and 18g of agar per liter, was used as the complete medium, and the synthetic agar medium containg 20g of dextrose, 3g of $(NH_4)_2SO_4$, 1g of KH_2PO_4, 0.5g of $MgSO_4 \cdot 7H_2O$, 10mg of $FeSO_4 \cdot 7H_2O$ and 18g of agar per liter, was used as the minimum medium. For the regeneration of protoplasts, the solid CM and MM include 44.7g of KCl(0.6M) per liter, was used.

Protoplast formation

Yeast cells grown in production medium to the log phase were harvested and washed twice with an aqueous solution of 0.9% NaCl. The cells were suspended in PTP buffer (99mM Tris, 860μM EDTA, and 50mM 2-mercaptoethanol, pH 8.0) and incubated at 30°C for 10min. In order to prepare protoplasts, the yeasts (5×10^8 cells) were washed twice with 1.2M KCl and resuspended in 4 ml of 0.6 M KCl solution containing 4mg of zymolyase 5,000 and 50mM of 2-mercaptoethanol. After 60 min incubation at 30°C, protoplasts obtained were washed and stored in a solution of 1.2 M KCl containing 20 mM $CaCl_2$.

Fusion and regeneration

Protoplast fusion was induced under the action of PEG. The parental protoplasts ($1\text{-}2 \times 10^8$ of each auxotroph) were mixed in an appropriate combination and centrifuged. The protoplasts were resuspended in 1 ml of a pre-warmed (30°C) solution of 30% (w/v) PEG MW 4,000 in 20 mM $CaCl_2$, pH 8.0, and incubated at 30°C for 10 min.

After dilution of PEG with 5 ml of hypertonic MM, protoplasts were centrifuged and washed twice with 1.2M KCl containing 20 mM $CaCl_2$. Serial dilutions of treated protoplasts were mixed with 10 ml of MM or CM containing 0.6 M KCl and 0.8% agar which was melted and maintained 42°C, and immediately poured onto agar plates of the same nutrient medium containing 0.6 M KCl.

Fusion frequency was calculated by dividing the number of colonies per milliliter appearing after 7 days at 30°C on hypertonic MM by the number of colonies per milliliter on hypertonic CM.

Results

Intraspecific and intergeneric fusion of yeast protoplasts

In order to investigate the intraspecific and intergeneric fusion frequency, we carried out protoplast fusion of *S. cerevisiae* and *C. tropicalis*, each carring auxotrophic requirement as genetic markers. Protoplasts, prepared from auxotrophic strains of *S. cerevisiae* and *C. tropicalis* under the

Table 2. Fusion frequency between protoplasts of auxotrophic strains of *S. cerevisiae*

Protoplast pairs	Colony on MM	Colony on CM	Fusion frequency
RSC113 (Lys$^-$, Val$^-$)			
xRSC111 (Glu$^-$, Lys$^-$)	–	2.6×10^6	–
xRSC87 (Ile$^-$)	4.0×10^2	5.3×10^6	7.55×10^{-5}
xRSC86 (Panto$^-$)	6.0×10^2	1.5×10^6	4.00×10^{-4}
RSC111 (Glu$^-$, Lys$^-$)			
xRSC87 (Ile$^-$)	6.0×10^2	4.1×10^6	1.46×10^{-4}
RSC60 (Folic$^-$, Panto$^-$)			
xRSC87 (Ile$^-$)	7.0×10^2	5.0×10^6	1.40×10^{-4}
RSC91 (Trp$^-$)			
xRSC87 (Ile$^-$)	4.3×10^3	3.1×10^6	1.38×10^{-3}

Fusion was achieved by mixing washed protoplasts ($1.0-2.0 \times 10^8$ of each auxotroph), treated them (30°C, 10 min) with 1.0 ml of 30% (w/v) PEG molecular weight 4,000 in 20 mM $CaCl_2$, pH 8.0.

Table 3. Fusion frequency between protoplast of auxotrophic strains of *C. tropicalis*

Protoplast pairs	Colony on MM	Colony on CM	Fusion frequency
RCT 167 (His$^-$, Leu$^-$)			
xRCT87 (Phe$^-$, Arg$^-$)	2.8×10^{2}	6.61×10^{6}	4.24×10^{-5}
xRCT 104 (Ala$^-$)	4.9×10^{2}	4.14×10^{6}	1.18×10^{-4}
xRCT 40 (Lys$^-$)	1.8×10^{2}	2.01×10^{6}	8.96×10^{-5}
RCT 144 (Lys$^-$, Leu$^-$)			
xRCT 84 (Ser$^-$, Trp$^-$)	1.2×10^{2}	6.58×10^{6}	1.82×10^{-5}
xRCT 104 (Ala$^-$)	3.0×10^{2}	7.45×10^{6}	4.03×10^{-5}
xRCT 67 (Val$^-$)	7.6×10^{2}	1.57×10^{6}	4.84×10^{-4}
RCT 104 (Ala$^-$)			
xRCT 40 (Lys$^-$)	9.1×10^{2}	2.67×10^{6}	3.41×10^{-4}
xRCT 67 (Val$^-$)	8.4×10^{2}	1.48×10^{6}	5.68×10^{-4}

Fusion was achived by mixing washed protoplasts ($1.0-2.0\times10^{8}$ of each auxotroph), treated them (30℃, 10 min) with 1.0 ml of 30% (w/v) PEG molecular weight 4,000 in 20 mM $CaCl_2$, pH 8.0.

optimized conditions given in Materials and Methods, were mixed to give intraspecific and intergeneric complementary combination. A solution of 30 % (w/v) PEG 4,000 containing 20mM $CaCl_2$ was added to induce agglutination and fusion. As shown in Table 2 and Table 3, each of the intraspecific fusion frequency of *S. cerevisiae* and *C. tropicalis* was distributed from 1.38×10^{-3} to 7.55×10^{-5} and 5.68×10^{-4} to 1.82×10^{-5}, respectively, depending on the combination of parental strains. With respect to intergeneric fusion, the frequency was in the range of 1.75×10^{-4} to 1.96×10^{-6}, with a maximum frequency between RSC 91(Trp$^-$)and RCT 104 (Ala$^-$)(Table 4).The intergeneric fusion frequency was 100 folds lower than intraspecific fusion frequency on average, but comparable with the results obtained in mold protoplasts.[5,25]

Effect of PEG on intergeneric protoplast fusion

Fig. 1 shows the dependence of fusion frequency on PEG concentration and molecular weight. Mixtures of the parental protoplasts of RSC 91(Trp$^-$)and RCT 104(Ala$^-$)were treated with various concentrations of PEG 4,000 or 6,000 in the presence of 20 mM $CaCl_2$. Although the concentration of PEG was not critical within limits, 30 % (w/v) PEG were effective regardless of molecular weight merely because they stabilized with the smallest reduction in the number of protoplasts regenerated on CM and MM. And protoplast aggregates composed of up to 20 protoplasts were observed after addition of PEG solution as appeared in Fig. 2, if PEG concentrations were used at 30 % (w/v) or higer. However, the concentrations below 20 % (w/v) caused lysis of protoplast. These results showed that the maximum frequency

Table 4. Fusion frequency between protoplasts of *S. cerevisiae* and *C. tropicalis* complementing auxotrophs

Protoplast pairs	Colony on MM	Colony on CM	Fusion frequency
RSC 131 (Arg$^-$, Orn$^-$)			
xRCT167 (His$^-$, Leu$^-$)	1.0×10^{1}	5.09×10^{6}	1.96×10^{-6}
xRCT144 (Lys$^-$, Leu$^-$)	4.0×10^{1}	4.18×10^{6}	9.60×10^{-6}
xRCT104 (Ala$^-$)	1.4×10^{2}	4.87×10^{6}	2.87×10^{-5}
xRCT40 (Lys$^-$)	2.1×10^{2}	2.49×10^{6}	8.42×10^{-5}
RSC139 (Thr$^-$, Cys$^-$)			
xRCT167 (His$^-$, Leu$^-$)	3.0×10^{1}	5.70×10^{6}	5.26×10^{-6}
xRCT144 (Lys$^-$, Leu$^-$)	6.0×10^{1}	4.80×10^{6}	1.25×10^{-5}
xRCT104 (Ala$^-$)	4.0×10^{1}	5.20×10^{6}	7.69×10^{-6}
xRCT40 (Lys$^-$)	2.7×10^{2}	3.36×10^{6}	9.04×10^{-5}
RSC91 (Trp$^-$)			
xRCT167 (His$^-$, Leu$^-$)	1.2×10^{2}	5.50×10^{6}	2.18×10^{-5}
xRCT144 (Lys$^-$, Leu$^-$)	1.5×10^{2}	3.00×10^{6}	5.00×10^{-5}
xRCT104 (Ala$^-$)	3.5×10^{2}	2.00×10^{6}	1.75×10^{-4}
xRCT40 (Lys$^-$)	2.9×10^{2}	4.32×10^{6}	6.72×10^{-5}
RSC87 (Ile$^-$)			
xRCT167 (His$^-$, Leu$^-$)	3.0×10^{1}	5.11×10^{6}	5.87×10^{-6}
xRCT144 (Lys$^-$, Leu$^-$)	2.0×10^{2}	3.20×10^{6}	6.25×10^{-5}
xRCT104 (Ala$^-$)	2.5×10^{2}	9.51×10^{6}	2.63×10^{-5}
xRCT40 (Lys$^-$)	2.0×10^{2}	9.01×10^{6}	2.22×10^{-5}

Fusion was achived by mixing washed protoplasts ($1.0-2.0\times10^{8}$ of each auxotroph), treated them (30℃, 10 min) with 1.0*ml* of 30% (w/v) PEG molecular weight 4,000 in 20 mM $CaCl_2$, pH 8.0, and eliminated PEG by two washes in 0.6 M KCl containing 20 mM $CaCl_2$. Protoplasts were plated onto regeneration minimal medium and complete medium to determine fusion frequency.

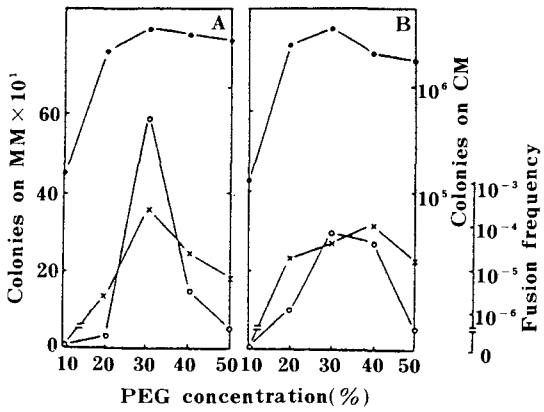

Fig. 1. Effect of PEG concentration on intergeneric fusion frequency between protoplasts of RSC 91(Trp-)and RCT 104(Ala⁻).

2.0×10^8 protoplasts of each auxotroph were treated with PEG 4,000 (A) and 6,000 (B) at different concentration, dissolved in 20 mM $CaCl_2$, pH 8.0 (30℃, 10 min). ○, colonies on regeneration MM; ●, colonies on regeneration CM; ×, fusion frequency.

of intergeneric protoplast fusion between protoplasts of *S. cerevisiae* RSC 91(Trp⁻)and *C. tropicalis* RCT 104(Ala⁻)was 2.3×10^{-4} at 30% PEG 4,000 and 1.0×10^{-4} at 40% PEG 6,000. So we adopted 30% PEG 4,000 as standard procedure in subsequent experiments.

Effect of pH on intergeneric protoplast fusion

To determine the optimum pH for intergeneric protoplast fusion, solutions of 30% (w/v) PEG 4,000 containing 20 mM

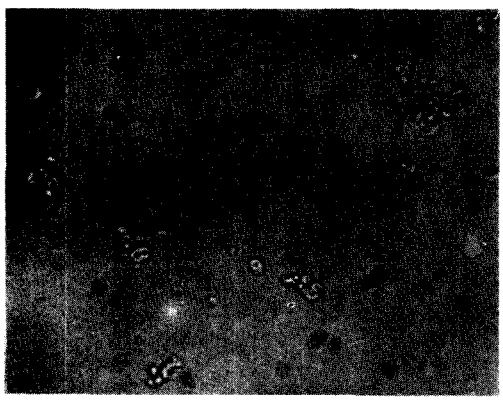

Fig. 2. Micrographs of protoplasts agglutinated under action of polyethylene glycol.

The protoplasts of *S. cerevisiae* and *C. tropicalis* were treated with PEG (MM 4,000, 30%)at 30℃ for (10min). Magnification: × 400.

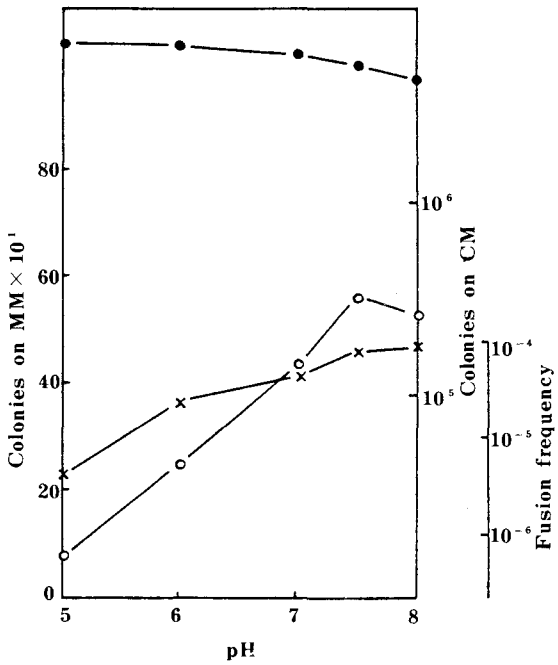

Fig. 3. Effect of pH on intergenic fusion frequency between protoplasts of RSC 91(Trip⁻) and 104(Ala⁻).

One ml of 30% (w/v) PEG 4,000 in 20 mM $CaCl_2$ at various pH levels was added to a mixture of 2.0×10^8 protoplasts of each auxotroph and incuabted at 30℃ for 10 min. ○, colonies on regeneration MM : ●, colonies on regeneration CM; ×, fusion frequency.

$CaCl_2$ were prepared at various pH and used for protoplast fusion of RSC 91 (Trp̄) and RCT 104 (Alā). Fig. 3 shows that fusion occurred in PEG solution at pH levels lower than 7.0, however, as the pH of PEG solution increased, the fusion frequency increased and was maximum at about 8.0. It should be pointed out that alkaline conditions had an important influence on intergeneric protoplast fusion, although PEG solutions above pH 7.0 were harmful causing reduced viability in the protoplasts.

Effect of temperature on intergeneric protoplast fusion

Protoplasts of RSC 91(Trp⁻)and RCT 104(Ala⁻)were fused in a solution of 30% (w/v) PEG 4,000 at different temperature. As appered in Fig. 4, fusion of protoplasts took place at 1°C, but increased with increasing temperature. And the optimum temperature for intergeneric protoplast fusion was 35°C. The number of regenerating protoplasts was steadily decreased and dramatically fell when incubated above 35°C.

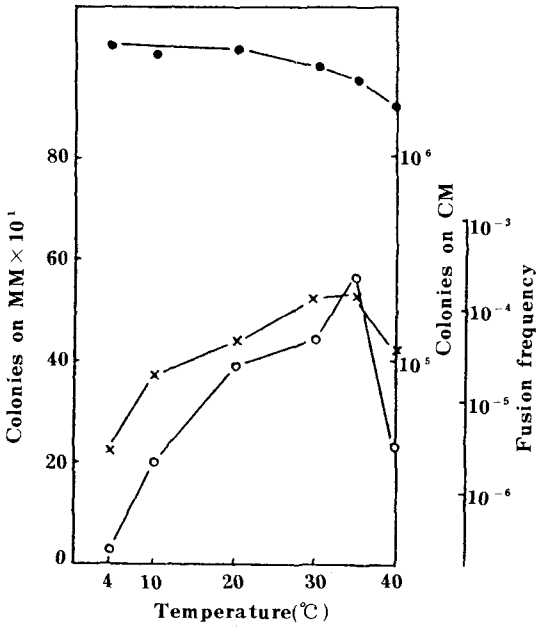

Fig. 4. Effect of temperature on intergeneric fusion frequency between protoplasts of RSC 91 (Trp⁻) and RCT 104(Ala).

One ml of 30% (w/v) PEG MW 4,000 pre-cooled and pre-warmed to proper temperature, was added to a mixture of 1.8×10^{8} protoplasts of each auxotroph, and incubated for 10 min at different temperatures. ○, colonies on regeneration MM; ●, Colonies on regeneration CM; ×, fusion frequency.

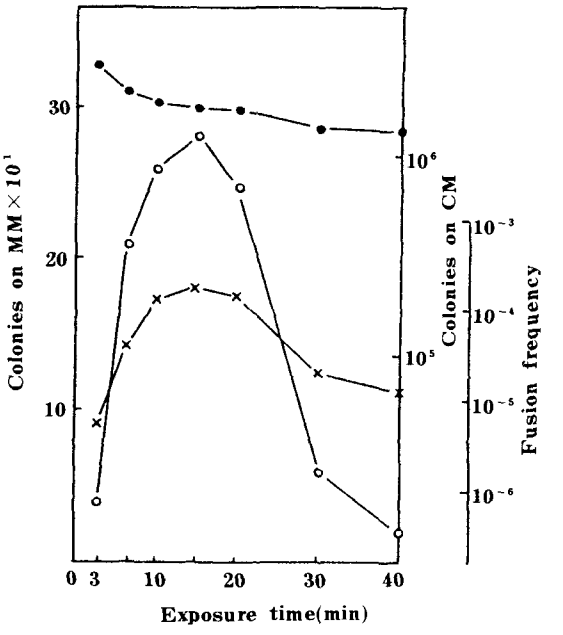

Fig. 5. Effect of exposure to PEG on intergeneric fusion frequency between protoplasts of RSC 91 (Trp⁻) and RCT 104(Ala⁻).

1.2×10^{8} protoplasts of each auxotroph were treated with 30% (w/v) PEG 4,000 at 30℃ for various intervals. ○, colonies on regeneration MM; ●, colonies on regeneration CM; ×, fusion frequency.

Effect of PEG exposure on intergeneric protoplast fusion

Results regarding relationship between PEG exposure time and intergeneric fusion frequency are shown in Fig. 5. The aggregation and fusion of protoplasts occurred immediately after PEG was assumed to have spread over the surface of the protoplasts. The fusion frequency reached a maximum level after 15 min of exposure to PEG. But above 20 min exposure, no further increase in fusion frequency was observed.

Effect of metal ions on intergeneric protoplast fusion

The effect of cations on fusion frequency was significant. Without cations such as Ca ions, dispite the good aggregation of protoplasts at higher PEG concentrations, the complementation frequency was negligible.[6] The influence of cations on intergeneric fusion frequency of yeast protoplasts was determined by using 30% (w/v) PEG 4,000 solution containing 10^{-3}M of various kinds of metal salts. Table 5 shows cations present in the PEG solution affected protoplast fusion, but had no influence on their viability. It can be seen that Ca or Zn ions as chloride promoted the highest level of fusion and approximately 100 folds more efficiently than PEG solution as control.

Table 5. Effect of metal ions on fusion frequency of intergeneric protoplast fusion between RSC91 (Trp⁻) and RCT 104(Ala⁻)

Metal ions (10^{-3}M)	Colony on MM	Colony on CM	Fusion frequency
$CaCl_2$	4.1×10^{2}	3.40×10^{6}	1.21×10^{-4}
$CuSO_4$	4.0×10^{1}	2.48×10^{6}	1.61×10^{-5}
$FeSO_4$	1.2×10^{2}	2.52×10^{6}	7.76×10^{-5}
$MgSO_4$	2.0×10^{2}	2.45×10^{6}	8.16×10^{-5}
$MnCl_2$	9.0×10^{1}	3.62×10^{6}	2.49×10^{-5}
$ZnCl_2$	2.8×10^{2}	2.42×10^{6}	1.16×10^{-4}
Control	1.0×10^{1}	2.79×10^{6}	3.58×10^{-6}

Protoplasts of each auxotroph were mixed in a 1 : 1 ratio and treated (30℃, 10 min) with 1.0 ml of a solution of 30% (w/v) PEG molecular weight 4,000, pH 8.0 containing various metal ions.

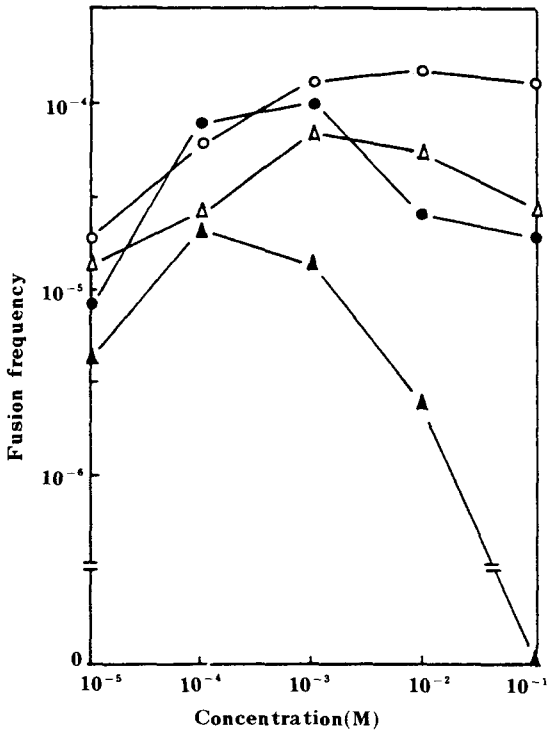

Fig. 6. Effect of metal ions concentration on intergeneric fusion frequency between protoplasts RSC 91(Trp⁻) and RCT 104(Ala⁻).

Protoplasts of each auxotroph were treated (30℃, 10 min) with 1.0 m*l* of PEG solution (4,000, 30%) containing various concentrations of metal ions. ○, $CaCl_2$; ●, $ZnCl_2$; △; $MgSO_4$; ▲, $CuSO_4$

In addition, the optimum concentration of cations for fusion frequency was also investegated, as appeared in Fig. 6. Cation concentrations at 10^{-5}M had no influence and gave the same fusion frequency as the PEG solution without any salt additon. And the fusion frequency was increased with increasing cation concentration to 10^{-3}M, but Cu ions as sulfate reduced fusion frequency to a minimum above 10^{-2}M, especially at 10^{-1}M it was completely inhibitory.

Discussion

Active interest in protoplast fusion as a method for strain improvement stemmed from the discovery of the fusogenic activity of the high molecular weight polyethylene glycol with plant protoplast systems.[1] This compound is now used universally in all microorganisms. In the majority of published reports, fusion experiments have utilized auxotrophic mutant strains, which after treatment with PEG are plated onto a minimal medium forcing the selection of fused protoplasts by nutritional complementation. The success of protoplast fusion for stable hybrid formation was directly concerned with the influences of various external parameters on aggregation and fusion of the parental protoplasts.[26] All varieties of PEG were able to stabilize and aggregate protoplasts and induce protoplast fusion at appropriate concentrations. PEG 4,000 and PEG 6,000 proved to be the most effective, but the concentration used was considerably critical. The protoplast stabilizing effects of PEG in the presence of 20 mM $CaCl_2$ were optimum at 30%, and below 20% its stabilizing effect was lost, resulting in protoplast lysis. At levels higher than 30% the PEG was hypertonic, caused protoplasts to shrink, and gave intensive aggregation and a lower frequency of fusion. PEG was also toxic at the higher concentrations. The efficency of PEG was enhanced by the pH of the medium and optimum at pH 8.0. High pH may change some characters and could increase interaction between negatively charged mambrane lipid and Ca ions.[25] Higher temperatures had probably a dual effect, increasing the viscosity of the PEG solution facilitating contact with protoplasts, and making the cytoplasmic membranes more fusogenic by an increase in membrane fluidity which might be significant in membrane fusion.[27] The incubation time of protoplasts in PEG solution must be long enough for an entire contact of PEG with the protoplast surface. Cations added to PEG solution basically influenced the frequency of protoplast fusion.[6,25] The efficiency of PEG was increased by the addition of Ca ions. On the other hand, as little as 1 mM cations was effective in stimulating the fusion process. Interestingly, with from 1 mM to 100 mM $CaCl_2$, the stimulating effect proved similar. Constabel and Kao have demonstrated the importance of Ca ions for increase in the fusion frequency in higher plant protoplasts.[28] They assumed that PEG acts as a molecular bridge between adjacent membranes, either directly by hydrogen bonds, or indirectly by Ca ions.

요 약

전분을 발효할 수 있는 새로운 효모균주의 개발을 목적으로 PEG를 fusogen으로 사용하여 이속 효모간 원형질체 융합을 시도하였다. 원형질체 융합에 사용한 친균주의 genetic marker로는 상보적 영양요구성을 이용하였고 각각의 영양요구성이 상호 보완되어 최소배지상에 자라는 colony를 융합체로 하였다. *S. cerevisiae*와 *C. tropicalis*의 이속간 원형질체의 융합빈도는 10^{-4}~10^{-6}정도였으며, 원형

질체 융합에 있어서 PEG 4,000, 30%가 가장 좋은 효과를 나타내었다. 최적 pH는 8.0이었으며, PEG의 처리효과는 온도가 상승함에 따라 증가하여 35℃에서 최적을 나타내었고 또한 Ca이온의 첨가에 의해서도 증가하였는데 Ca이온의 촉진효과는 1 mM과 100 mM 농도 사이에서 최고를 나타내었다.

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