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Production of Red Pigment by Using Protoplast Fusion of *Monascus anka*

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Monascus 속간의 원형질체 융합에 의한 적색색소의 생산

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Abstract

This study aims at producing red pigment effectively by using protoplast fusion. Auxotrophic mutants of *M. anka*, 4478-27-37(*Thi⁻*, *Met⁻*), 4478-27-62(*Thi⁻*, *Arg⁻*) were derived from *M. anka*, 4478-27. *M. anka*, 6540-185-24 (*Pan⁻*, *Leu⁻*) and *M. anka*, 6540-185-72 (*Arg⁻*) were derived from *M. anka*, 6540-185. The optimal conditions of protoplast fromation were at time by using mixtures of chitinase (100mg/m*l*), cellulase (5mg/m*l*) and β-glucuronidase (5mg/m*l*). The protoplast of those auxotrophic mutants were fused effectively, in the solution of 30% PEG 6,000, 0.01M-$CaCl_2$, 0.05M-glycine, pH 6.0. Fusion frequencies were 0.70%-0.85%. Fusants, No. 14 and 42 produced highest red pigment among them.

Key words: red pigment, auxotrophic mutants, *Monascus anka*, protoplast fusion

Introduction

Protoplast fusion is of current interest because it provides a new and useful method for the fundamental and applied genetics. Since the use of polyethyleneglycol (PEG) to induce the fusion of plant protoplast efficiently[1,2], this technique has been successfully applied for the fusion of animal cells[3], yeast[4-7], and bacteria[8,9]. As regards fungi, the first attempt at the controlled protoplast fusion and application for breeding of the strains has been reported to increase yield of enzyme and fermentation. The subject concerning crosses between two mutants strains derived from wild strains of fungi such as *Asp. nidulance, Asp. niger, Asp. oryzae,* and *Asp. sojae* was first reported by Roper and Pontecorvo[10,11]. Uchida, *et. al*[12] have demonstrated that the parasexual life cycle of *Asp. oryzae* and *Asp. sojae* was available for the improvement of these industrial strains. Furthermore, there are many reports concerning intraspecific and interspecific fusion of filamentous fungi including *Asp.* sp[13,14] and *Penicullium,* sp.[15,16].

Although the improvement effect was not so significant as expected, some results have been achieved by using the fusion of fungi[17]. Recently, Furuya, *et. al*[18] already succeeded in the isolation of fusant producing protease and amylase over two times as much as the parent srains. The present purpose is to imrove the red pigment productivity of the strains. We examined conditions for the formation, fusion and regeneration of protoplasts and established procedures for obtaining fusant between different mutant strains of *M. anka,* 4478-27 and *M. anka,* 6540-185.

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Materials and method

Microorganisms

Auxotrophic mutants of *M. anka,* 4478-27-37 and *M. anka,* 6540-185-62 derived from *M. anka,* 4478-27 and *M. anka,* 6540-185. This mutants induced by repeatedly MNNG treatment in our provious report[19].

Medium

Complete medium (CM) contained 10% glucose, 1.0% peptone, and 2.0% agar, pH 5.0 and minimum medium (MM) contained 10% glucose, 1.0% potato, 0.15% $NaNO_3$, 0.1% $MgSO_4$ $7H_2O$, 0.25% KH_2PO_4 and 2.0% agar. Pigment producing medium (PM) contained 3.0-5.0% steamed rice powder, 0.15% $NaNO_3$, 0.1% $MgSO_4$ $7H_2O$, 0.25% KH_2PO_4 and 3.0% sweet potato, or 3.0% cassava[20,21].

Isolation of auxotrophic mutants

Auxotrophic mutants were concentrated by a method similar to that of Woodward[22] and Alderberg, *et.al*[23]. The mutagenized spores were suspended in the liquid MM at a concentration of $10^6/ml$ and incubated on the rotary shaker at 30℃ for 4-5 days. The cultures were filtered through glass wool filter to remove germings of parental cells and ungerminated spore in the filterates were collected by centrifugation and resuspended by fresh medium. Cultivation and filteration were repeated 4-5 times, and then the spores were plated on the CM agar medium. The density of spores ($1.5\times10^7/ml$) was reduced to about $2\times10^3/ml$ by this procedure. Colonies appeared after incubation for 3 days at 30℃ in which transferred to fresh CM agar plates. The resulting colonies were checked for growth on the MM agar medium. Substances required by these mutants were determined by transferring them to various supplemented media. To characterize their growth factor, the nutritional requirements were determined by observing the growth response on minimal media supplemented with either amino acid pool, vitamins or nucleic acid bases[24].

Protoplast formation

Protoplast were prepared from all strains using the procedure described by Anne[16], Ferenczy, *et. al*[13] with some modification. Mycelium growth in CM was harvested on Whatman No. 1 filter paper and washed with 0.6M KCl. For protoplast formation, 100mg mycelium was suspended in 1m*l* of 0.6M-KCl solution containing appropriate concentration of enzymes such as chitinase (250unit/mg, Sigma Co.), cellulase (Onozuka, R-10, Kinki Co.) or β-glucuronidase (1100unit/mg, Sigma Co.). After incubation at 30℃ for 4 hr with gently shaking, liberated protoplast were seperated from mycelial debris by filteration through a sintered glass filter and washed with 0.6M -KCl, twice.

Protoplast fusion

Protoplast fusion were carried out by the method of Anne, *et. al*[15]. Protoplast ($1.0\times10^6/ml$) of each auxotroph from the different strains used were mixed and centrifugated at 1,000 rpm for 10 min. The pellets protoplast were suspended in 1m*l* of a prewarmed (30℃) solution of a various concentration of PEG in appropriate molarity of $CaCl_2\cdot2H_2O$, 0.05M-glycine, pH 6.0 and incubated at 30℃ for 10 min. After fusion mixture centrifuged at 1,000 rpm for 10 min, this pellet was diluted with 6.0m*l* of 0.6M-KCl and centrifuged again, and then washed with 0.6N-KCl, twice. Serial dilutions of treated protoplasts were plated at suitable dilution on MM to selected nutritionally complementing fused protoplasts and on CM.

Fusants transferred 3 times on MM for stabilization by using toothpick. Colonies derived from fusion products were maintained on MM. The fusion frequency was calculated from the ratio colonies development on MM and CM.

Regeneration of protoplasts with solid CM or MM made by hypertonic with 0.6M-KCl

Estimation of pigment by fusants

Pigment extraction from pigment producing medium was carried out according to in our previous report[19].

Results and discussion

Isolation of auxotrophic mutants

Approximately 600 mutants derived from *M. anka*, 4478-27 and *M. anka*, 6540-185 were isolated by a toothpick technique.

According to the number of different nutritional factors required by complementary nutrients, auxotrophic mutants of each cultures is shown in Table 1. Auxotrophic mutants requiring simultaneously amino acid and vitamins were classified as single or double markers. 11 different mutants were found 7 amino acids and 2 vitamins requirments as nutritional markers. Mutants of *M. anka*, 4478-27 -37 (*Thi*$^-$, *Met*$^-$) and 4478-27-62 (*Thi*$^-$, *Arg*$^-$) were derived from 4478-27. *M. anka*, 6540-185-24 (*Pan*$^-$, *Leu*$^-$) and 6540-185-72 (*Arg*$^-$) were derived from 6540-185. They showed higher production of pigment. From these data, these mutants described above were used for protoplast fusion.

Protoplast formation

The cell wall of *Monascus anka* contains chitosan, β-1,3 glucan as the main components in contrast to most of other filamentous fungi which contain chitin and β-1,3 glucan as main cell wall components[25]. In order to make the protoplast of *M. anka* 4478-27-37 (*Thi*$^-$, *Arg*$^-$), *M. anka* 4478-27 -62 (*Thi*$^-$, *Arg*$^-$), and to form the protoplast of *M. anka* 6540-185-24 (*Pan*$^-$, *Leu*$^-$) and 6540-185-72 (*Arg*$^-$), cell walls were lysed by using enzymes such as chitinase, cellulase and β-glucuronidase. It is important to design the construction of protoplast formation with lytic enzymes.

These kinds of lytic enzyme and mixtures of their concentration and combination were examined to make for the protoplast. Mixtures of chitinase, cellulase and β-glucuronidase were examined in 0.01M-sodium phosphate buffer, pH 6.0.

Fig. 1 showed the test about concentration and combination of lytic enzymes. When enzymes mixtures of chitinase (10mg/m*l*), cellulase (5mg/m*l*) and β-glucuronidase (5mg/m*l*) were used the highest level of protoplast formation were 5.0 10^5/m*l* while incubated at 30℃ for 4h. When chitinase or cellulase was used respectively, a small

Table 1. Auxotrophic mutants derived from M. anka 4478-27 and 6540-185

Strains No.	Auxotrophs	Pigment (O.D. 500nm)	Origins
M. anka, 4478-27		0.67	
M. anka, 4478-27-28	*Thi*$^-$	0.72	*M. anka*, 4478-27
M. anka, 4478-27-37	*Thi*$^-$, *Met*$^-$	0.80	*M. anka*, 4478-27
M. anka, 4478-27-62	*Thi*$^-$, *Arg*$^-$	0.77	*M. anka*, 4478-27
M. anka, 4478-27-86	*Leu*$^-$	0.68	*M.anka*, 4478-27
M. anka, 6540-185		0.70	
M. anka, 6540-185-9	*Thi*$^-$, *Lys*$^-$	0.80	*M. anka*, 6540-185
M. anka, 6540-185-11	*Thi*$^-$	0.74	*M. anka*, 6540-185
M. anka, 6540-185-24	*Pan*$^-$, *Leu*$^-$	0.82	*M. anka*, 6540-185
M. anka, 6540-185-41	*Thi*$^-$, *Pro*$^-$	0.76	*M. anka*, 6540-185
M. anka, 6540-185-72	*Arg*$^-$	0.84	*M. anka*, 6540-185

Thi: Thiamin, Pan: Ca-panthotenate,
Met: Methionine, Leu: Leucine, Arg: Aginine,
Lys: Lysine, Pro: Proline

These mutants were incubatd for pigment production with each complemented reguired nutrients at 30°C for 10 days. Culture broth were extracted with 80% alcohol and were filterated and then were measured at 500nm.

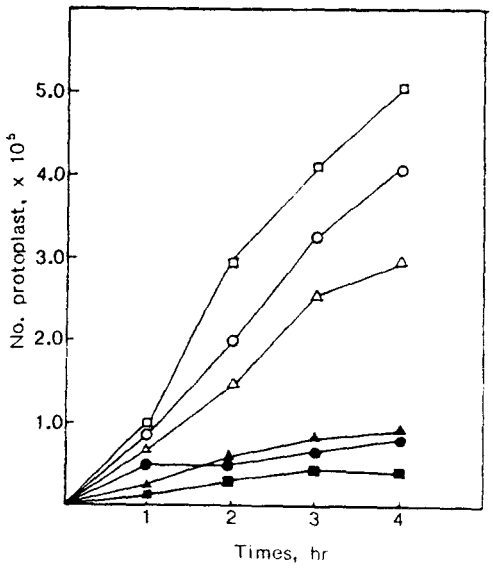

Fig. 1. Effects of various concentration of chitinase, cellulase and glucuronidase on the formation of protoplasts by mutant of M. anka, 4478-27-37.
○——○: 10mg/ml chitinase, 5mg/ml cellulase, 10mg/ml glucuronidase
□——□: 10mg/ml chitinase, 5mg/ml cellulase, 5mg/ml glucuronidase
△——△: 5mg/ml chitinase, 10mg/ml cellulase, 5mg/ml glucuronidase
●——●: 10mg/ml chitinase,
▲——▲: 10mg/ml cellulase,
■——■: 10mg/ml glucuronidase

amount of protoplast were released. Mixtures of chitinase (5mg/ml), cellulase (10mg/ml) and β-glucuronidase (5mg/ml) increased slightly the protoplast formation. The level of its formation was released 3.0×10^5/ml.

Peberdy and Issac[14] indicated that lytic enzymes were related to formation of mold protoplast. Kevei, *et. al*[26] reported that they performed to make the protoplast of *Aspergillus nidulans* with lytic enzyme obtained from *Trichoderma harzianum* culture broth. Nohmi, *et. al*[27] made protoplast by enzymes which had been made from mixtures of enzymes of snail digestive tract and cellulase. On the other hand, Ohnuki, *et. al*[28] established procedures obtaining protoplast from *Mucor* sp. by using chitosanase, chitinase and sulfatase. From our results, the mixed enzymes produced more protoplast than a single enzyme.

Effect of pH

Protoplast formation was influenced by pH during treatment of enzymes. For this experiment, pH was adjusted ranging from 5.0 to 9.0 and enzymes were tested for protoplast formation with the adjusted pH. As shown in Fig. 2, the maximum yield of protoplast was obtained at pH 6.0 by using the mixtures of those enzymes.

Effect of osmotic stabilizers

To select osmotic stabilizers or protoplast formation, various concentration of KCl, $MgSO_4\cdot7H_2O$ or sorbitol were examined as osmotic stabilizer for protoplst formation. As shown in Fig. 3, KCl appeared to be more effective than another stabilizer, and the optimal concentration of KCl were at the ranging from 0.4 to 0.6M. The increase of over 0.6M KCl caused a marked decrease in the proto-

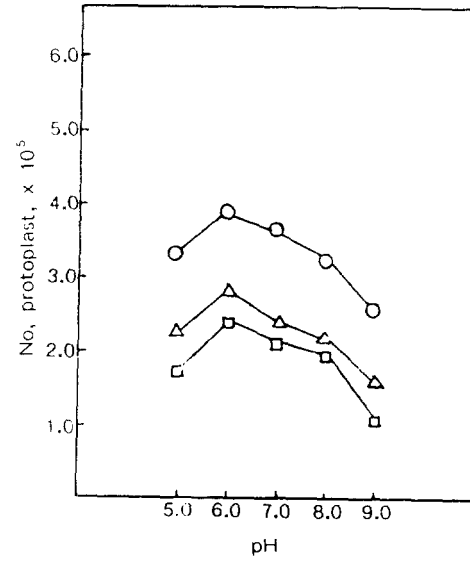

Fig. 2. Effects of pH on the protoplasts formation by mutant of M. anka,4478-27-37. Reaction mixtures was used 0.01M sodium phosphate buffer.
□——□, incubation for 1h; △——△, incubation for 2h; ○——○, incubation for 3h.

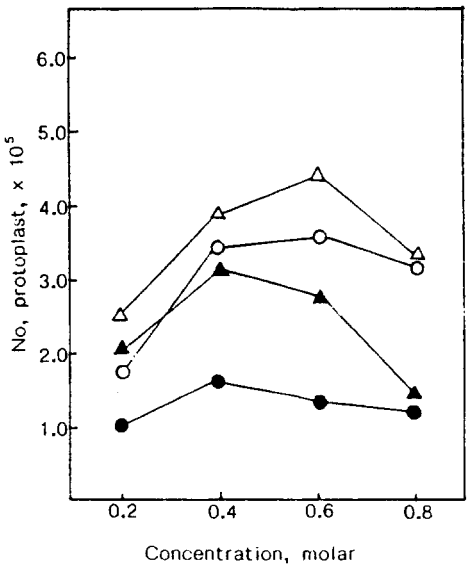

Fig. 3. Effects of different osmotic stabilizers on the formation of protoplasts by mutant of *M. anka*, 4478-27-37.
△——△, KCl; ○——○, Mannitol; ▲——▲, Sorbitol; ●——●, $MgSO_4 \cdot 7H_2O$.00

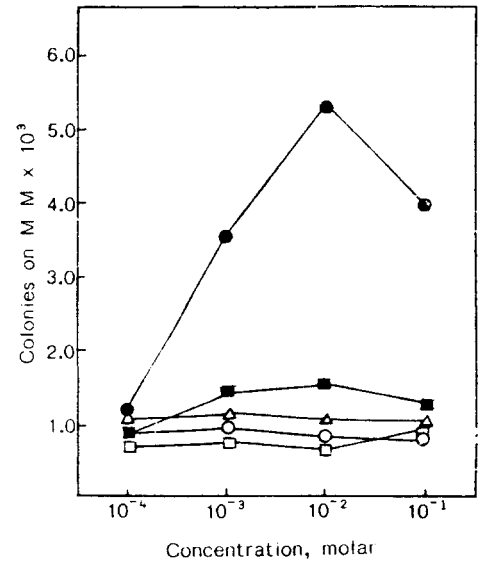

Fig. 4. Effects of different ions on the yield of protoplast fusion by mutant *M. anka*, 4478-28-37, developing on MM.
Each protoplasts of auxotrophs (1.0×10^6/m*l*) were treated with 1m*l* of solution of 30% (w/v) PEG 6,000, 0.05M-glycine, pH 6.0 containing each concentrations of different ions at 30°C for 30 min
●——●, $CaCl_2 \cdot 2H_2O$; △——△, KCl; ■——■, $MgSO_4 \cdot 7H_2O$; ○——○, $NaNO_3$; □——□, NaCl.

plast yield. It was also reported that 0.6M-KCl used as osmotic stabilizer were most effective to form the protoplast of *Asp. awamori* and *Asp. oryzae*[17].

Factors affecting protoplast fusion

Cations present in the PEG solution affect protoplast fusion of various organisms. As shown in Fig. 4, 0.01M $CaCl_2$ was optimal concentration and also promoted the highest level of fusion for protoplast formation at pH 6.0. On the other hand, Mg^{2+} of $MgSO_4 \cdot 7H_2O$ was stimulated the fusion the lower than 0.01M-$CaCl_2$, and the reason is that Mg^{+2} inhibited the fusion of protoplast. The increasing of concentration of Na^+ or K^+ reduced protoplast fusion to the degree of minimum 0.1M-KCl or 0.1M-$NaNO_3$ almost completely inhibited fusion.

Constabel and Kao[29] have demonstrated that Ca^{2+} increased the amount of fusion yield in higher plant protoplasts.

They assumed that PEG reacted as a molecular bridge between adjacent membranes, either directly by hydrogen bonds, or indirect by Ca^{2+}. On the other hand, the concentration of PEG plays important role in the protoplast fusion[1,10].

M. anka, 4478-27-37 (*Thi*$^-$, *Met*$^-$) and 4478-27-62 (*Thi*$^-$, *Arg*$^-$), and *M. anka*, 6540-185-24 (*Pan*$^-$, *Leu*$^-$), 6540-185-72 (*Arg*$^-$) were fused too by using PEG. The extent of protoplast aggregation was measured at various PEG concentration by counting apparent number of regenerating colonies. As shown in Fig. 5, protoplast aggregates composed of up to 15 protoplasts were observed after addition of PEG, if PEG concentration were used at 30%, but the concentration of lower than 30% was a week stabilizer to make protoplast fusion.

30% PEG 6,000 was optimal concentration to fuse the protoplast between both strains. In view of results above, fusion between protoplast of

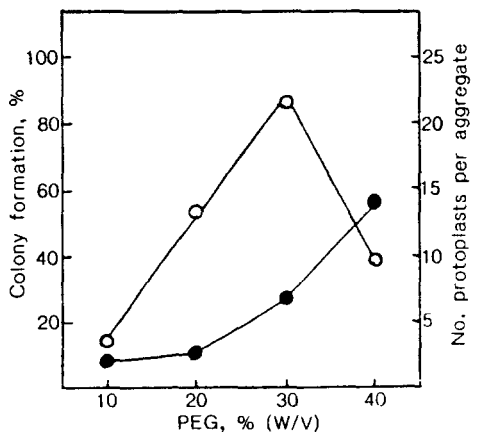

Fig. 5. Effects of PEG concentration on aggregation of protoplasts by mutant of *M. anka*, 4478-27-37. The number of protoplasts per aggregate(○) was calculated from the number of protoplasts before PEG treatment and colony counts(●) with normalization by protoplast regeneration ratio

nutritionally complementing strains was detected by formation of heterokaryons developing on solid MM. Colonies appeared from PEG after incubation for 72h or 96h, unfused protoplasts, spore and mycelium are unable to develope these media. As shown in Table 2, the range of the frequency of fusion, based on the ratio of the number of colonies developing on MM and on CM after PEG treatment was 0.70 to 0.85%. Keller and Melchers[30] demonstrated that heterokaryon formation were found after treatment of mixing auxotrophic protoplast with polyethyeneglycol 4,000 and calcium ions at high pH. Kouni and Satoi[17] suggested that the frequency of the fusion between *Asp. awamori* and *Asp. oryzae* was 0.5%-0.9%. Anne[15] indicated that fusion frequency of *Penicillum* sp. was 0.1%-0.7%. From these data, the present data were in accordance with other reports[17,21].

Fusion of protoplast by auxotrophic mutants

The fusion experiment between protoplasts of serveral auxotrophic mutants was performed. Table 3 showed the pigment productivities by fusants. 12 fusnats were selected from among 300 isolates. They were obtained from the fusion of *M. anka*, 4478-27-37 with 6540-185-24, *M. anka*, 4478-27-37 with 6540-185-72, *M. anka*, 4478-27-62 with 6540-185-24, *M. anka*, 4478-27-62 with 6540-185-72. These fusants were allowed to produce the pigments on their production medium at 30℃ for 10 days. Two fusants, No. 42 and 14 showed highest pigment productivity among fusants which

Table 2. Fusion frequency between protoplasts of auxotrophic mutants obtained from *Monascus anka*, 4478-27 and 6540-185

Fusion mixtures		Protoplast regenerated on CM		Fusants formed on MM		Fusion frequency°(%)
		Before fusion treatment	After PEG treatment	Before fusion treatment	After fusion treatment	
M. anka,	4478-27-37	1.20×10^6	6.4×10^4	0	1.04×10^3	0.85
	×					
	6540-185-24	1.41×10^6	5.8×10^4			
M. anka,	4478-27-37	1.02×10^6	7.7×10^4	0	1.12×10^3	0.70
	×					
	6540-185-72	1.05×10^6	8.3×10^4			
M. anka,	4478-27-62	1.12×10^6	6.9×10^4	0	1.05×10^3	0.73
	×					
	6540-185-24	1.03×10^6	7.4×10^4			
M. anka,	4478-27-62	1.30×10^6	6.0×10^4	0	1.01×10^3	0.77
	×					
	6540-185-72	1.00×10^6	7.1×10^4			

Fusion were carried out by protoplast fusion in 30% PEG 6, 000, 0.01M-$CaCl_2$, and 0.05M-glycine, pH 6.0 with each complementary nutrients required of auxotrophic mutant at 30°C, for min. The frequency was defined as the ratio of the number of colonies growing on MM and CM agar.

Table 3. Pigment productivities by fusants

Fusion mixtures		Fusant, No	M.D.W. (g)	Pigment production (O.D. 500nm)
M. anka,	4478-27-37 x 6540-185-24	12	0.285	0.70
		42	0.270	0.88
		62	0.280	0.80
M. anka,	4478-27-37 x 6540-185-72	8	0.254	0.68
		53	0.266	0.70
		68	0.250	0.73
M. anka,	4478-27-62 x 6540-185-24	22	0.240	0.85
		36	0.290	0.80
		48	0.280	0.78
M. anka,	4478-27-62 x 6540-185-72	14	0.277	0.89
		56	0.246	0.86
		86	0.255	0.80

M.D.W. : Mycelium dry weight

These fusants were incubated with pigment production media containing 3.0% steamed rice, 1.0%seasme extract, 0. 1% $MgSO_4 \cdot 7H_2O$, 0.25% KH_2PO_4 and each complementary nutrients on the rotay shaker at 30° for 10 days. Culture broth were diluted 10 times with 80% alcohol and were filterated and then were measured at 500*nm*.

were measured at 500*nm*. Generally, interspecific and intraspecific fusants obtained from mold showed higher enzyme productivity than their parent strains[17,18]. Therefore, the pigment producing ability of No. 14 and 42 mutants was slightly higher than that auxotrophic mutants. It is assumed that the fusants increased the pigment production under the same conditions.

요 약

Monascus 균주의 적색색소의 최적 생산조건을 규명하기 위하여, *Monascus anka* IFO 4478과 IFO 6540의 영양요구성 돌연변이주인 *M. anka*, 4478-27-37 (*Thi*$^-$, *Met*$^-$), 4478-27-62 (*Thi*$^-$, *Arg*$^-$)와 *M. anka*, 6540-185-24 (*Pan*$^-$, *Leu*$^-$), 6540-185-72 (*Arg*$^-$)의 균주를 이용하여 세포융합을 시도하였다.

Protoplasts의 생성은 chitinase (10mg/m*l*), cellulase (5mg/m*l*) 및 β-glucuronidase (5mg/m*l*)의 혼합처리가 효과적이었고, 융합조건은 protoplasts를 30% PEG 6,000, 0.01M-$CaCl_2$ 및 0.05M-glycine, pH 6.0으로 처리했을 때 융합빈도는 0.7%-0.85%이였다. 융합주 중에서 No. 14와 42의 색소생산능이 가장 높았다.

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