α -Tocopherol Production by an Analog-resistant Strain of *Euglena gracilis* Z

Yoshiki TANI and Shin-oi OSUKA

Research Center for Cell and Tissue Culture, Faculty of Agriculture,
Kyoto University, Kyoto 606, Japan

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Improvement of the α -tocopherol productivity of *Euglena gracilis* Z was achieved by derivation of an analog-resistant strain. Among various compounds tested, β -2-thienylalanine was selected as the best inhibitor for derivation of high α -tocopherol-producing strains.

A number of β -2-thienylalanine-resistant strains were obtained on UV irradiation with or without streptomycin treatment, and also on spontaneous mutation. One strain, S-T1, produced 4 times as much α -tocopherol as the wild type strain.

As the result of optimization of the culture conditions, strain S-T1 produced 48.4 mg per l of culture broth or 1.8 mg per gram of dry cell weight (DCW) of α -tocopherol intracellularly. The addition of homogenisate and ethanol increased the production to 130.3 mg per l or 4.8 mg per gram of DCW. Finally, a high amount of α -tocopherol, 180.4 mg per l of 6.3 mg per gram of DCW, was obtained with the feeding of glucose during the cultivation.

Euglena gracilis has been used as a useful unicellular organism for studies on photosynthesis, chloroplast development and other metabolic processes because its chloroplast development and replication are subject to environmental control.^{1~3)} In fact, most mutants of *E. gracilis* reported have had mutations related to the chloroplasts. Such mutations affect the colony color and photosynthetic function.^{4,5)} However, few studies have been reported on the production of useful substances by *E. gracilis* and no mutant strain for improved production has appeared.

In a previous paper, we reported the α -tocopherol production by *E. gracilis* Z.⁶⁾ The *de novo* biosynthetic pathway for tocopherols is known to be connected to the shikimate pathway, and quinone and aromatic amino acid biosyntheses.⁷⁾ Thus, the presence of a variety of regulation mechanisms for α -tocopherol production could be assumed.

In this study, we developed a process for the mutagenization of *E. gracilis* Z, and isolated a strain which is resistant to β -2-thienylalanine and which produces a higher amount of α -tocopherol than the wild type strain. To our

knowledge, this is the first report of a strain of *E. gracilis* of which the productivity of a metabolite was improved by mutagenization. The optimization of the culture conditions for α -tocopherol production by the strain was also investigated.

Materials and Methods

Microorganisms and chemicals. *E. gracilis* Z, which was selected as an α -tocopherol-producer,⁶⁾ was used as the parent strain for the derivation of mutant strains. Authentic tocopherol samples were kind gifts from Dr. Y. Wakiguchi, Eizai Co., Ltd. All other chemicals were obtained from usual commercial sources.

Medium and cultivation. Koren and Hutner's (KH) medium⁸⁾ was used as the basal medium for maintenance, the mutation process and comparison of the α -tocopherol productivities of mutant strains. Solid medium for the isolation of mutant strains was prepared by adding 1~1.2% (w/v) of agar to the KH medium (pH 5.5). The α -tocopherol production medium for strain S-T1 was modified on the basis of the optimal medium for α -tocopherol production reported in the previous paper,⁶⁾ and consisted of 3.5 g glucose, 1.5 g peptone Polypepton, Nihon Seiyaku), 0.1 g KH_2PO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 mg thiamin-HCl, 0.5 g cyanocobalamin, 5 mg $\text{Na} \cdot \text{EDTA}$, 5 mg $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 1.8 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and

2.5 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 100 ml of tap water, pH 5.0. Cultivation was carried out as described in the previous paper⁶⁾ except for the main culture, for which 15 ml of medium was placed in a 500-ml shaking flask.

Analytical methods Growth and the intracellular amount of α -tocopherol were determined as described in the previous paper.⁶⁾

Isolation of analog-resistant mutants.

1) *UV irradiation with or without streptomycin treatment* Cells were harvested at the mid or late logarithmic phase, washed and then suspended in 10 mM potassium phosphate buffer, pH 7.0. UV irradiation was carried out in a dish containing 10 ml of the cell suspension (10^5 cell/ml) with continuous stirring.⁴⁾ The UV dose was $300 \mu\text{W}/\text{cm}^2$ on the surface of the cell suspension.

During the course of mutagenization as described above, it was observed that UV irradiation caused the loss of chloroplasts by *E. gracilis* and that green cells had a higher ability as to tocopherol production than bleached cells. Nicolas and Nigon reported a process for the production of plastidial antibiotic-resistant mutants involving UV irradiation.⁹⁾ This led to the employment of streptomycin treatment after UV irradiation for enrichment of green colonies: the cell suspension was exposed to UV light for 20, 40 or 60 sec ($60 \sim 180 \text{ J}/\text{m}^2$, which is far from the lethal dose) and then inoculated into 50 ml of the medium (pH 8.0) containing streptomycin at a final concentration of $500 \mu\text{g}/\text{ml}$ in a 500-ml shaking flask.⁹⁾ The flask was incubated for 3 or 6 days under ordinary conditions, except for the dark, to prevent photoreactivation. After the treatment, the cells were harvested, washed two times and then suspended in 10 mM potassium phosphate buffer, pH 7.0.

Cell suspension after UV irradiation with or without streptomycin was pipetted onto the solid medium contain-

ing 0.003%, 0.006% or 0.01% of an inhibitor. The number of cells inoculated onto a plate was about 10^5 . The plates were incubated at first in the dark for 5~7 days and then under illumination. More than 1-month incubation in the light was necessary to obtain the maximal number of green colonies.

2) *Spontaneous mutation.* Cells were inoculated into 50 ml of the medium in a 500-ml shaking flask containing the inhibitor at a low concentration, i.e., 0.003% β -2-thienylalanine, and then incubated under the ordinary conditions. After 7~10 days the cells were transferred to each new medium. The process was repeated for 5 months. The concentration of the inhibitor in the medium was gradually increased, finally to the lethal concentration, i.e., 0.025% of β -2-thienylalanine.

To determine the inhibitor-resistance of cells, cells were harvested, washed and diluted in 10 mM potassium phosphate buffer, pH 7.0, to $10^2 \sim 10^3$ cells/ml, and then 0.3 ml of the suspension was plated on the solid medium containing the inhibitor at the final concentration of 0.05%. Colonies of an inhibitor-resistant strain were obtained after 10~14 days incubation under illumination.

Results and Discussion

Derivation of an analog-resistant strain

Compounds inhibiting the growth of *E. gracilis* Z were screened from among a number of analogs related to the biosynthesis of tocopherols. The inhibition by β -2-thienylalanine, ethionine and 5-fluorophenylalanine was reversed by the addition of homogentisate or α -tocopherol (Fig. 1). Therefore, a number of strains resistant to these compounds were derived through UV irradiation and the spon-

Fig. 1. Growth Inhibition of the Wild Type Strain by Analogs and Its Reversal by Homogentisate or α -Tocopherol.

Cultivation was carried out in KH-medium containing lethal concentration of β -2-thienylalanine (T), ethionine (E) and 5-fluorophenylalanine (F), without (○) or with the addition of homogentisate (△) or α -tocopherol (●).

Table I. α -TOCOPHEROL PRODUCTIVITIES OF RESISTANT STRAINS

Strains	Analog (‰)	Number of strains						
		ng	Amount of α -tocopherol produced					
			<1.0	1.0~1.5	1.5~2.0	2.0~3.0	3.0~4.0	4.0<
Green strains								
With UV treatment	None		6	2				
	T ²⁾ (0.003~0.01)		15	7				
	E ³⁾ (0.003~0.01)		13	9	1	1		
	F ⁴⁾ (0.003~0.01)	1	3	2				
With UV and Sm ¹⁾ treatment	T (0.003~0.01)		14	5	2			
	E (0.003~0.01)		2	3				
	F (0.003~0.01)		2	2				
Spontaneous mutation	T (<0.025)		16	19	1	10	2	1
	E (<0.03)		20	17	8	5		
	F (<0.02)		13	6	3	1	2	
White strains		15	12	5				

Cultivation was carried out in KH-medium in test tube for 5~8 days. The amount of α -tocopherol was expressed relative to the value obtained with the wild type strain cultivated concurrently in each experiment. Sm,¹⁾ streptomycin; T,²⁾ β -2-thienylalanine; E,³⁾ ethionine; F, 5-fluorophenylalanine. ng, no growth.

taneous mutation process. The lethal concentrations of the inhibitors in the solid culture were much lower than those in the liquid culture. Table I shows the α -tocopherol productivities of these resistant strains. Among them, spontaneously-derived resistant strains showed higher α -tocopherol productivities than those obtained through UV irradiation. A β -2-thienylalanine-resistant strain, S-T1, obtained through the spontaneous mutation process was chosen as the best α -tocopherol producer. Through the strain retained the α -tocopherol productivity and resistance of β -2-thienylalanine during the periodic transfer, it is still only tentatively characterized as an analog-resistant mutant. β -2-Thienylalanine, and analog of phenylalanine, is a strong growth of inhibitor and was reported to inhibit the activity of 2-keto-3-deoxy-D-arabino-heptulosonate 7-phosphate synthase.¹⁰⁾

Strain S-T1 produced about 4-times as much α -tocopherol as the wild type strain in a test tube culture for 8 days. The amount of α -tocopherol produced in the culture was 15.6 mg/l of culture broth and 0.75 mg/g-dry cell weight (DCW).

White colonies, which comprised the majority on the plate after UV irradiation,

showed slow or no growth and low α -tocopherol productivity. Sigeoka *et al.* reported that white strains of *E. gracilis* Z produced a lower amount of α -tocopherol under light culture conditions than green strains did, the distribution of α -tocopherol in green cells being 23.5% in chloroplasts and 46.8% in mitochondria.¹¹⁾ It was also reported that the biosynthesis of tocopherols was closely related to chloroplasts.^{12~15)} The UV irradiation in the present study could have damaged principally chloroplastic DNA, attacking the point related to chloroplast formation. This might be the reason for the low productivity of α -tocopherol by green strains, in spite of enrichment of green clones with the streptomycin treatment.

Optimization of the culture conditions for α -tocopherol production by strain S-T1

Concentrations of glucose and peptone. The optimal medium for α -tocopherol production by the wild type strains of *E. gracilis* Z,⁶⁾ in which strain S-T1 produced 12.06 mg/l of culture broth or 0.96 mg/g-DCW α -tocopherol, was modified as to the glucose and peptone concentrations (Fig. 2).

The cell yield increased slightly with an

Fig. 2. Effects of the Glucose and Peptone Concentrations on α -Tocopherol Production by Strain S-T1.

Cultivation was carried out in the α -tocopherol production medium for wild type strain with 2.5% glucose (A), 3.0% glucose (B) or 3.5% glucose (C).

increase in the glucose concentration. Glucose at the concentration of 3.5% was better than at 3.0% and 2.5%, with any concentration of peptone, for the α -tocopherol production. Glucose at 4% strongly inhibited both growth and α -tocopherol production. Glucose at 3.5% and peptone at 0.9% was found to be best combination in this experiment. The cell yield was better with an initial pH of 6~7 than 5, but the α -tocopherol production was highest at pH 5 and steeply decreased at pH 6. The pH of the culture broth was almost constant at 4.0 during the cultivation when 0.9% of peptone was employed, and increased with an increase in the peptone concentration (Fig. 2). This suggested that the pH of the culture broth is closely related to α -tocopherol production. Considering the NH_4^+ formed from excess prptone, pH adjustment was carried out during the cultivation, though no significant difference was observed on pH adjustment. An increase in the MgSO_4 concentration from 0.1% to 0.2% was effective as to the α -tocopherol production, among modifications of other component concentrations.

Effect of aeration. A change in aeration during the cultivation affected both the α -tocopherol production and cell yield (Fig. 3). The highest value was obtained at $R=0.03$. Under this condition, the pH of the culture broth remained lower, and the combination of 3.5% glucose and 1.5% peptone gave higher α -tocopherol production than 3.5% glucose and

Fig. 3. Effect of Aeration on α -Tocopherol Production by Strain S-T1.

500-ml shaking flasks containing 15, 30, 50 and 100 ml of the α -tocopherol production medium for strain S-T1 were inoculated with a final concentration of 5.0% (v/v) of cells. R, ratio of the volume of medium to the total capacity of the flask.

0.9% peptone.

Since the cultivation was carried out under the light, an illumination effect might be related to the higher α -tocopherol production with a decreased volume of the culture medium.

Effects of additives. The addition of precursors of α -tocopherol increased the α -tocopherol productivity of the wild type strain.⁶⁾ At any concentration, L-tyrosine was not so effective for the α -tocopherol production by strain S-T1. Homogentisate at the concentration of 0.1% increased the α -tocopherol productivity, but more than 0.2% caused steep

Table II. THE EFFECTS OF ADDITIVES ON α -TOCOPHEROL PRODUCTION BY STRAIN S-T1

Additives (%)	Strain S-T1			Wild type strain		
	Growth	α -Tocopherol	Final Ph	Growth	α -Tocopherol	Final pH
None	100	100	6.9	100	100	6.0
L-Tyrosine (0.01)	102	82	7.2	94	126	6.0
Homogentisate (0.1)	98	124	5.9	86	240	4.7
Ethanol (0.8)	121	139	5.7	106	124	5.9
L-Tyrosine (0.01), homogentisate (0.1) and ethanol (0.8)	149	138	4.5	105	206	4.7

Cultivation was carried out in the α -tocopherol production medium for the wild type strain supplemented with each compound for 10~12 days under illumination. The amounts of α -tocopherol and cell yields are relative.

decreases in both the α -tocopherol production and growth. Table II compares the effects of additives on the α -tocopherol productivities of the wild type and resistant strains. Ethanol at the concentration of 0.8% had a good effect on the α -tocopherol production and cell yield. It seemed that the effective additives tend to bring the pH of the culture broth down. Besides, L-ascorbate, AMP, ADP, ATP and several detergents caused steep decreases in both growth and the α -tocopherol production.

Effect of feeding. Glucose was fed when the glucose concentration in the medium dropped below 0.5%. The amount of α -tocopherol produced remarkably increased. Feeding of glucose and peptone at the final concentrations of 2% and 0.5%, respectively, increased the cell yield but decreased the α -tocopherol production. The good result with glucose-feeding might be partly due to the maintenance of the pH of the culture broth at 4.0~4.5. Feeding of 3.5% glucose and 1.5% peptone led to higher α -tocopherol production than that of 3.5% glucose and 0.9% peptone.

Feeding of ethanol also had a good effect on α -tocopherol production. Feeding of glucose and ethanol or homogentisate resulted in lower production than glucose-feeding. In the case of feeding of ethanol and peptone at the final concentrations of 0.2% and 0.5%, the α -tocopherol production was lower than that with no feeding.

When homogentisate and ethanol were added to the medium initially, and then glu-

Fig. 4. Effect of Glucose Feeding on α -Tocopherol Production by Strain S-T1.

During cultivation in the α -tocopherol production medium for strain S-T1 (A), 2.0% glucose, final concentration, was fed at the times indicated by arrows (B).

cose, ethanol or the mixture was fed, the amount of α -tocopherol was the same as in the case of no feeding and lower than that with glucose-feeding. In this case, the pH of the culture broth decreased to 4.0.

As shown in Fig. 4, strain S-T1 produced 48.4 mg/l culture broth or 1.8 mg/g-DCW α -tocopherol when cultured as described under **Materials and Methods**, and the amount of α -

Table III. IMPROVEMENT OF α -TOCOPHEROL PRODUCTION BY STRAIN S-T1

Medium	Cell yield	α -Tocopherol produced		Final pH
	(g/l)	(mg/l)	(mg/g-DCW)	
KH-medium (Wild type)	23.9	4.5	0.2	
(S-T1)	20.8	15.6	0.75	
α -Tocopherol production medium for wild type strain	12.6	12.1	1.0	6.9
α -Tocopherol production medium for strain S-T1	26.8	48.4	1.8	5.8
Feeding of glucose	29.0	180.4	6.3	4.3
Feeding of glucose and homogentisate	29.0	132.8	4.6	3.9
Homogentisate and ethanol added	27.6	129.2	4.7	4.3
Feeding of glucose and ethanol	27.1	130.3	4.8	3.9

tocopherol reached 180.2 mg/l culture broth or 6.3 mg/g-DCW with the feeding of only glucose two times. Thus, the high α -tocopherol production by strain S-T1 was achieved without expensive additives, and it seemed to be related to the pH. The improvement of the α -tocopherol production by *E. gracilis* Z as the result of mutagenization and optimization of the culture conditions are summarized in Table III.

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