Optimized Conditions for High Erythritol Production by *Penicillium* sp. KJ-UV29, Mutant of *Penicillium* sp. KJ81

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Abstract To improve the erythritol productivity of *Penicillium* sp. KJ81, mutants were obtained using UV irradiation and NTG treatment. Among these mutants, *Penicillium* sp. KJ-UV29 revealed no morphological changes, yet was superior to the wild strain in the following three points: (1) *Penicillium* sp. KJ-UV29 produced more erythritol than the wild strain under the same conditions, (2) no foam was produced during cultivation, unlike the wild strain, and (3) the mutant produced a significantly lower amount of glycerol. *Penicillium* sp. KJ-UV29 produced as much as 15.1 g/L of erythritol, whereas the wild-type *Penicillium* sp. KJ81 only produced 11.7 g/L. *Penicillium* sp. KJ-UV29 only generated 6.1 g/L of glycerol, compared to 19.4 g/L produced by the wild strain. When investigating the optimal culture conditions for erythritol production by the mutant strain *Penicillium* sp. KJ-UV29, sucrose was identified as the most effective carbon source, and the mutant was even able to produce erythritol in a 70% sucrose-containing medium, although a 30% sucrose medium exhibited the highest productivity. The production of erythritol by *Penicillium* sp. KJ-UV29 was also significantly increased by the addition of ammonium carbonate, potassium nitrate, and sodium nitrate. Accordingly, under optimal conditions, *Penicillium* sp. KJ-UV29 produced 45.2 g/L of erythritol in a medium containing 30% sucrose, 0.5% yeast extract, 0.5% $(NH_4)_2C_2O_4$, 0.1% KNO_3, 0.1% $NaNO_3$, and 0.01% $FeSO_4$ with 1 vvm aeration and 200 rpm agitation at 37°C for 7 days in a 5-L jar fermentor.

Keywords: chemical Warfare, detoxification, prolidase, organophosphorous compound

INTRODUCTION

Erythritol is a sugar alcohol that has about 70~80% of the sweetness of sucrose. Widely distributed in nature [1], erythritol has been detected in fruits, lichens, and mushrooms, and also found in small amounts in the body fluids of mammals [2-5]. Furthermore, since erythritol is nontoxic to humans, it has been studied as an alternative to sugar, due to its low calorific value and noncarcinogenic nature [6].

The production of erythritol by microorganisms had already been studied in *Moniliella tomentosa* var. *pollinis*, *Candida zeylanoides*, *Trichonosporonoides* sp., and *Aureobasidium* sp. [7-9]. However, on an industrial scale, there have been relatively few studies on erythritol production using microorganisms. Wako *et al.* [10] previously reported on the industrial production of erythritol by *Aureobasidium* sp. SN-115, a mutant strain of *Aureobasidium* sp. SN-124 [11]. This mutant could produce 180 g/L of erythritol in a medium containing 40% glucose, and was found to be superior to the wild strain as follows. First, the mutant did not produce any foam during cultivation, unlike the wild strain, second, the mutant produced 47.6% erythritol in a medium containing 22.5% glucose, compared to 41.8% obtained with the wild strain, and third, when the glucose concentration in the medium increased from 22.5% to 47%, the erythritol production by the wild strain drastically declined from 41.8% to 14.2%.

Previously, the current authors reported on the isolation and identification of *Penicillium* sp. KJ81, a soil isolate that exhibits a high production of erythritol [12], and investigated the optimal conditions for the production of erythritol by *Penicillium* sp. KJ81 [13].

Accordingly, the current study mutated *Penicillium* sp. KJ81 using conventional UV and NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) treatments and obtained a mutant(KJ-UV29) that produced significantly more erythritol than the wild strain.

MATERIALS AND METHODS

Microorganisms

The *Penicillium* sp. KJ81, an erythritol producer, was isolated from corn shock soil, as previously reported by the current authors [12]. The mutant strains, *Penicillium*

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Table 1. Production of erythritol and glycerol by *Penicillum* sp. KJ81 and its mutants

Strain	Erythritol production (g/L)	Glycerol production (g/L)
Penicillium sp. KJ81 (wild type)	11.7	19.4
Penicillium sp. KJ-UV29 (mutant)	15.1	6.1
Penicillium sp. KJ-UV27 (mutant)	14.9	17.6

sp. KJ-UV27 and KJ-UV29, were both derived from *Penicillium* sp. KJ81 and exhibited high erythritol production.

Mutagenesis and Mutant Isolation

The wild strain *Penicillium* sp. KJ81 was grown in Medium A (10% sucrose, 0.5% yeast extract) at 30°C for 3 days with rotary shaking at 150 rpm, then diluted to approximately 1 × 10^7 cell/mL with 0.85% NaCl solution and poured into a Petri dish. The suspensions were irradiated with a 20 W UV lamp (Sankyu Denki, Japan) at a distance of 50 cm for 30 min at room temperature. Next, the irradiated suspensions were spread on Medium B (40% sucrose, 0.5% yeast extract, 1.5% agar) and incubated at 30°C for 2 days, then the fast-growing colonies were selected and inoculated into Medium C (50% sucrose, 0.5% yeast extract) and incubated at 30°C on a rotary shaker at 150 rpm. Mutants were then selected on the basis of a faster growing rate and further analyzed for their erythritol productivity. In addition, the selected strains were incubated with a 0.1 M citrate buffer (pH 5.5) containing 1 mg/mL NTG for 40 min at room temperature and immediately washed with Medium A (10% sucrose, 0.5% yeast extract). Mutants were then selected from the NTG-treated cell suspension on the same basis of a faster growing rate and further analyzed for their erythritol productivity.

Culture Conditions

The finally selected mutant was inoculated into a test tube (16 × 150 mm) containing 5 mL of Medium D (30% sucrose, 0.5% yeast extract) and incubated at 30°C for 4-6 days with rotary shaking at 200 rpm. The seed culture (5 mL) was then transferred into a 250 mL Erlenmeyer flask containing 100 mL of Medium G at 30°C for 4-6 days with rotary shaking at 200 rpm. The culture was centrifuged at 8,000 rpm for 15 min, then the supernatant was filtered through a 0.22 μm membrane filter and used as a sample for analyzing the amount of erythritol and glycerol.

The fermentation experiment was performed using a 5-L jar fermentor (Korea Fermentor Co., Korea). The seed culture (100 mL) was transferred to a 5-L jar fermentor containing 3 L of an optimal medium, including 30% sucrose, 0.5% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, and 0.05% $MgCl_2$ (pH 7.0) and grown in the same optimal medium for 2 days at 37°C. The temperature and pH in the fermentor were controlled at 37°C and 7.0, respectively, the aeration rate was 1 vvm, and the agitation speed was controlled at 200 rpm. During cultivation, 10% AZ-20R was added to the optimal medium as an anti-foaming agent.

Analytical Procedures

The production of erythritol was determined by paper chromatography and high performance liquid chromatography (HPLC). For a qualitative analysis, the paper chromatography was run in a descending direction on Whatman filter paper No. 1 for 16 h with a solvent system of ethylacetate : 2-propanol : water (6 : 3 : 1). For a quantitative analysis, the HPLC was performed using a Gilson model 712 equipped with a Rainin NH_2 column. The mobile phase was acetonitrile-water (3 : 1), the flow rate 1.0 mL/min, and the products detected using an RI detector.

RESULTS AND DISCUSSION

Isolation of Mutants

To improve the erythritol productivity of the soil isolate *Penicillium* sp. KJ81, cell suspensions were treated with a 20 W UV lamp at a distance of 30 cm for 30 min. The UV-irradiated suspensions were then plated on Medium B (40% sucrose, 0.5% yeast extract, 1.5% agar) and the plates incubated at 30°C for 2 days. Approximately 300 colonies exhibiting the fastest growth rate were then selected and cultivated in Medium C (50% sucrose, 0.5% yeast extract). In addition, about 30 of the selected colonies were analyzed in regard to their erythritol production using HPLC. Finally, the two mutants that produced the highest amount of erythritol were selected and designated as KJ-UV27 and KJ-UV29. The erythritol yield of these two mutants was 30% higher than that of *Penicillium* sp. KJ81, the wild strain (Table 1). Unexpectedly, NTG treatment of the two mutants did not increase their erythritol production. Furthermore, since *Penicillium* sp. KJ-UV29 produced the highest amount of erythritol as well as the lowest amount of glycerol as a by-product, *Penicillium* sp. KJ-UV29 was selected for further characterization.

Effect of Carbon Sources

The production of erythritol by strain KJ-UV29 was examined in the presence of various carbon sources. To

Table 2. Effect of carbon sources on production of erythritol by *Penicillium* sp. KJ-UV29

Carbon source	Erythritol production (g/L)
Sucrose	15.1
Glucose	14.4
Galactose	13.9
Maltose	4.7
Mannose	3.3
Fructose	2.9
Lactose	4.5
Mannitol	-*
Sorbotol	-
Arabinose	-
Xylose	-

Cultivation was carried out for 5 days at 30°C in a medium containing 10% of each carbon source and 0.5% yeast extract.
*, not determined

find a suitable carbon source for erythritol production, the mutant strain was grown in a medium containing 0.5% yeast extract as the growth factor and various carbon sources. Each carbon source was added to the medium at a concentration level of 10% (w/v). As shown in Table 2, *Penicillium* sp. KJ-UV29 was able to use sucrose, glucose, galactose, maltose, mannose, fructose, and lactose as carbon sources, which matched the results previously reported by Hajny *et al.* [8] and Wako *et al.* [14]. However, among the carbon sources tested, sucrose was identified as the most effective substrate for erythritol production and chosen as the carbon source for subsequent experiments. Meanwhile, *Penicillium* sp. KJ-UV29 was unable to use arabinose or xylose as a carbon source, as they inhibited completely cell growth.

Effect of Sucrose

To investigate the effect of various concentrations of sucrose as the most efficient carbon source, the erythritol and glycerol production by *Penicillium* sp. KJ-UV29 was studied over a sucrose concentration range of 30 to 70% (w/v). As shown in Fig. 1, the highest cell growth and erythritol production of *Penicillium* KJ-UV29 were observed with a sucrose concentration of 70% (w/v). Yet, in the case of growth at concentrations of sucrose higher than 40%, the production of glycerol as a by-product sharply increased, which was similar to the results of Wako *et al.* [14]. Thus, the optimum concentration of sucrose for erythritol production was determined at about 30%.

Effect of Yeast Extract

The effect of the yeast extract concentration on erythritol production was examined in Medium D with 30% sucrose as the carbon source. Fig. 2 shows that the mutant KJ-UV29 did not produce erythritol without any yeast extract, and the maximum amount of erythritol was obtained with an initial yeast extract concentration of 1.0%. At concentrations higher than 1.0%, the increasing rate of glycerol production was much higher than that of erythritol production, indicating that the addition of yeast extract facilitated and enhanced glycerol production. Therefore, the optimum yeast extract concentration was determined to be 0.5%.

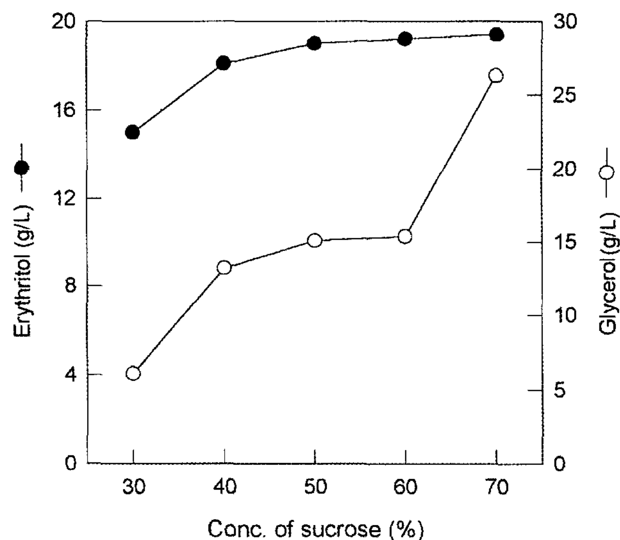

Fig. 1. Effect of sucrose concentration on erythritol production by *Penicillium* sp. KJ-UV29.

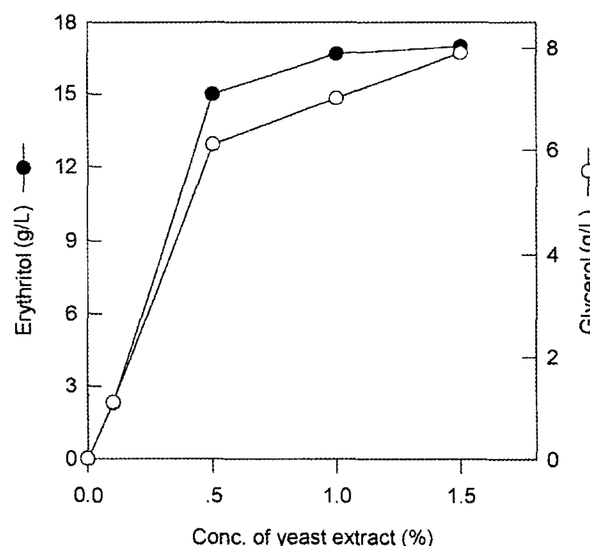

Fig. 2. Effect of yeast extract concentration on erythritol production by *Penicillium* sp. KJ-UV29.

Effect of Inorganic Salts

The effect of various inorganic salts was investigated

Table 3. Effect of inorganic salts on production of erythritol and glycerol by *Penicillium* sp. KJ81 and KJ-UV29

Inorganic salt	*Penicillium* sp. KJ81		*Penicillium* sp. KJ-UV29	
	Production of erythritol (g/L)	Production of glycerol (g/L)	Production of erythritol (g/L)	Production of glycerol (g/L)
None	11.5	21.7	15.0	6.1
$(NH_4)_2C_2O_4$	2.5	4.7	39.5	18.1
$(NH_4)_2SO_4$	24.1	32.4	7.2	6.0
$NaNO_3$	14.3	26.1	27.2	16.3
Na_2HPO_4	6.7	12.4	11.7	6.2
KH_2PO_4	7.4	14.9	7.1	4.9
K_2HPO_4	1.8	2.9	12.5	9.2
$MgCl_2$	6.9	11.9	13.1	9.1
KNO_3	22.1	42.0	42.8	20.2
$FeSO_4$	7.3	13.6	10.3	3.6
$ZnSO_4$	2.2	4.3	5.1	3.6
KCl	14.0	28.1	17.8	8.3
$AlCl_3$	2.0	4.4	10.2	4.4

0.5% inorganic salts were added to Medium D.

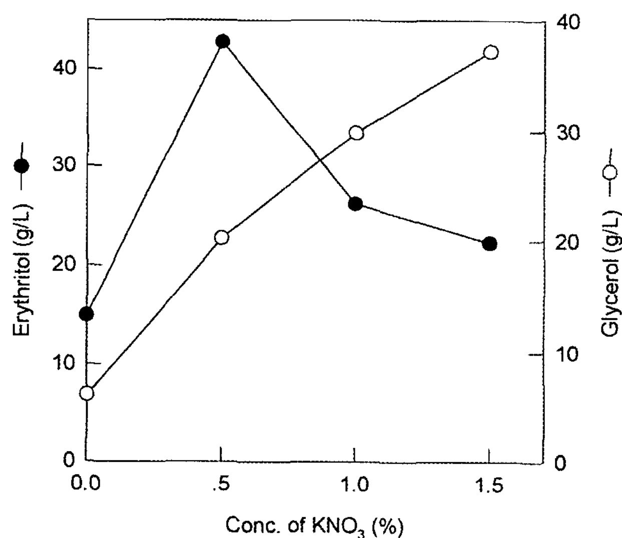

Fig. 3. Effect of KNO_3 concentration on erythritol production by *Penicillium* sp. KJ-UV29 in Medium D.

using Medium D based on the addition of 0.5% of each inorganic salt. Table 3 shows that the erythritol production was improved by the presence of $(NH_4)_2C_2O_4$, KNO_3, and $NaNO_3$. Among the inorganic salts, KNO_3 was identified as the most effective for erythritol production by *Penicillium* sp. KJ-UV29, which was different from the results for *Penicillium* sp. KJ81, the wild strain. Subsequently, the effect of the KNO_3 concentration on erythritol production was investigated. Fig. 3 shows that the highest amount of erythritol production was obtained in a concentration of 0.5% (v/w) KNO_3, yet with more than 0.5% KNO_3 concentration, the erythritol production was gradually reduced. Therefore, the optimum KNO_3 concentration for erythritol production was determined to be 0.5% (w/v).

Effect of pH and Temperature

The effect of environmental factors on erythritol production was also examined. As such, KJ-UV29 was grown in Medium D adjusted to various pH values by 0.1 N NaOH/ 0.1 N HCl for 5 days before measuring the amount of erythritol and glycerol. As shown in Fig. 4, the maximum erythritol production was at pH 7.0, the productivity of erythritol was inhibited with strong acidic and alkaline pHs, and an alkaline pH led to an increase of glycerol production. Therefore, the optimum pH for erythritol production was determined to be 7.0, which was different from the optimum pH (pH 5.6) for *Aureobasidium* sp. reported by Wako *et al.* [14] and the optimum pH (pH 4-5) for *Monitiolla tomontosasms* reported by Hajny *et al.* [8].

To find the optimal culture temperature, KJ-UV29 was cultivated at 30°C, 35°C, 40°C, and 45°C. Fig. 5 shows that the maximum erythritol production was at 37°C. Also, the pattern of glycerol production was similar to that of erythritol production, except that the production of glycerol reached a maximum level at 35°C, then decreased at 37°C. Therefore, the maximum temperature

Table 4. Production of erythritol and glycerol by wild- and mutant-strain under different culture conditions

Strain	Culture condition		Production (g/L)	
			Erythritol	Glycerol
Penicillium sp. KJ81	Basal culture (a) in flask		11.7	21.6
	Optimal culture (b)	in flask	38.7	52.4
		in fermentor	28.2	43.1
Penicillium sp. KJ-UV29	Basal culture in flask		15.1	6.1
	Optimal culture (c)	in flask	60.1	29.3
		in fermentor	45.2	20.7

(a): basal medium consisting of 30% sucrose and 0.5% yeast extract.
(b): medium supplemented with 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, and 0.01% $MgCl_2$.
(c): medium supplemented with 0.5% $(NH_4)_2C_2O_4$, 0.1% KNO_3, 0.01% $NaNO_3$, and 0.01% $FeSO_4$.

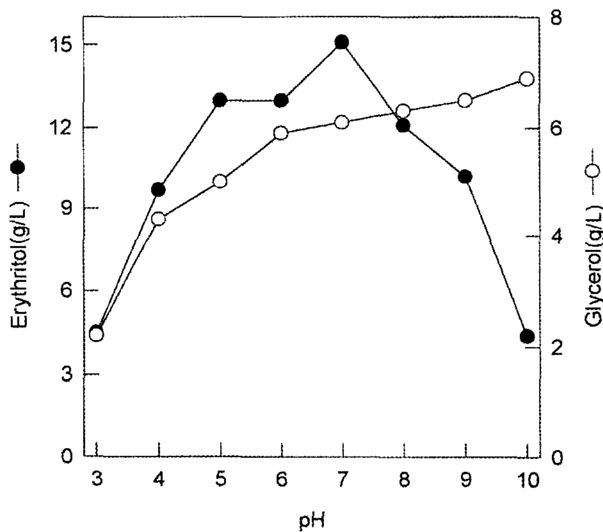

Fig. 4. Effect of initial medium pH on erythritol production by *Penicillium* sp. KJ-UV29 in Medium D.

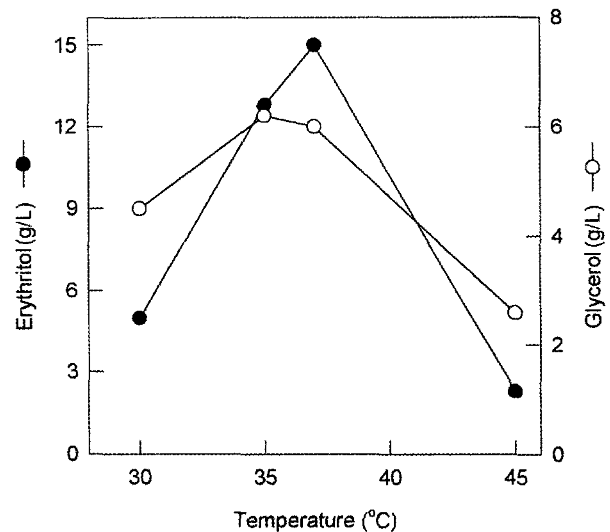

Fig. 5. Effect of temperature on erythritol production by *Penicillium* sp. KJ-UV29 in Medium D.

for erythritol production was determined to be 37°C. Since a rise in temperature led to an increase of glycerol production, which matched the results of Wako *et al.* [14], the production of glycerol was apparently proportionally related to cell growth.

Conditions of Fermentation

To investigate erythritol production in a jar fermentor, fermentation was carried out in a 5-L jar fermentor containing 3 L of the optimal medium (30% sucrose, 0.5% yeast extract, 0.5% $(NH_4)_2C_2O_4$, 0.5% KNO_3, 0.1% $NaNO_3$, and 0.01% $FeSO_4$). The erythritol production by *Penicillium* sp. KJ-UV29 increased for the first 6 days of fermentation, then remained nearly constant thereafter (Fig. 6). The cultivation time required for the maximum production of erythritol by *Penicillium* sp. KJ-UV29 was shorter than that required by *Penicillium* sp. KJ81. However, as shown in Table 4, the erythritol production in the fermenter was about 40% less than that in a flask. Finally, under optimum conditions, *Penicillium* sp. KJ-UV29

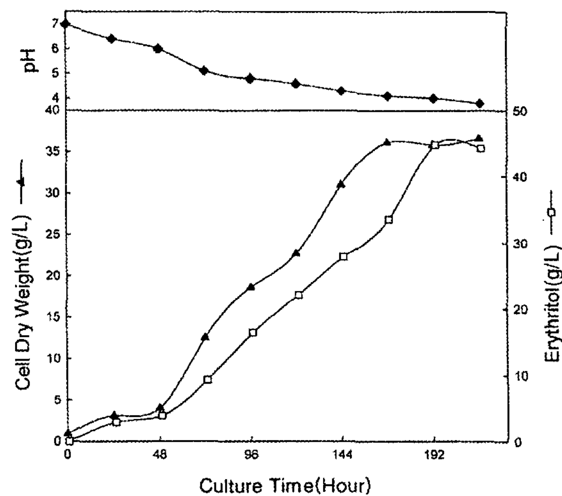

Fig. 6. Erythritol fermentation by *Penicillium* sp. KJ-UV29. Cultivation was performed in medium containing 30% sucrose, 0.5% yeast extract, 0.5% $(NH_4)_2C_2O_4$, 0.5% KNO_3, and 0.01% $FeSO_4$ at 37℃ with stirring at 200 rpm and aeration rate of 1 vvm.

produced 45.2 g/L of erythritol, which was almost twice the amount (28.2 g/L) produced by *Penicillium* sp. KJ81, the wild strain (data not shown). The culture pH was initially set at 7.0, yet decreased slowly throughout the fermentation. The production of glycerol continued to increase during the fermentation, however, the amount of glycerol produced by KJ-UV29 was still 40% lower than that produced by *Penicillium* sp. KJ81, the wild strain. In particular, with the addition of $FeSO_4$, there was a remarkable reduction in the amount of foam produced by *Penicillium* sp. KJ-UV29, thereby eliminating the need for an anti-foam agent. Accordingly, *Penicillium* sp. KJ-UV29, a mutant derived from *Penicillium* sp. KJ81, exhibited a significantly improved erythritol production and reduced glycerol and foam production compared to the wild strain.

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