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Increased erythritol production in fed-batch cultures of *Torula* sp. by controlling glucose concentration

D-K Oh[1], C-H Cho[2], J-K Lee[2] and S-Y Kim[2]

[1]*Department of Bioscience and Biotechnology, Sejong University, 98 Kunja-Dong, Kwangjin-Ku, Seoul 143-747, South Korea;* [2]*BioNgene, 307-1 Songsan-Ri, Yangkam-Myun, Hwasung-Kun, Kyongki-Do 445-930, South Korea*

The effect of glucose concentration on erythritol production by *Torula* sp. was investigated. The maximum volumetric productivity of erythritol was obtained at an initial glucose concentration of 300 g l^{-1} in batch culture. The volumetric productivity was maximal at a controlled glucose concentration of 225 g l^{-1}, reducing the lag time of the erythritol production. A fed-batch culture was established with an initial glucose concentration of 300 g l^{-1} and with a controlled glucose concentration of 225 g l^{-1} in medium containing phytic acid as a phosphate source. In this fed-batch culture, a final erythritol production of 192 g l^{-1} was obtained from 400 g l^{-1} glucose in 88 h. This corresponded to a volumetric productivity of 2.26 g l^{-1} h^{-1} and a 48% yield. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 248–252.

Keywords: erythritol; *Torula* sp.; fed-batch culture; glucose concentration; phytic acid; production rate

Introduction

Erythritol is a four-carbon polyol used as a food ingredient like other polyols with similar properties, including xylitol, sorbitol, mannitol, maltitol, lactitol, and isomalt [1]. It is a naturally occurring substance and is widely distributed in nature [3]. Erythritol is a metabolite or storage compound in seaweed and fungi, and fruits like melons, grapes, and pears also contain erythritol. It occurs frequently in fermented food, including wines and beers, and in processed vegetables, such as soy sauce and oriental miso bean paste [12–16].

Erythritol is a moderately sweet bulking agent, with 60–70% of the sweetness of sucrose in a 10% solution. Erythritol has a substantial negative heat when dissolved in a solution, providing a strong cooling effect. Since erythritol is almost as sweet as sucrose, but has no bitter aftertaste, it can be used in combination to improve the taste of intense sweeteners that have a bitter aftertaste, such as aspartame [3].

Erythritol can be produced by microbial methods using osmophilic yeasts and some bacteria [2,10]. It has been produced industrially using a mutant of *Aureobasidium* [5], which produced erythritol with a volumetric productivity of 1.82 g l^{-1} h^{-1} and a yield of 43.8% in a medium containing 40% glucose. This is the highest reported erythritol productivity and yield among erythritol-producing microorganisms.

The *Torula* sp. used in this study was isolated from a 40% sucrose solution [6,7], and the erythritol production of the strain was improved with supplemental Mn^{+2} and Cu^{+2} [8]. Using *Torula* sp., we determined the optimum range of glucose concentration for effective erythritol production and attempted to increase the erythritol production rate by controlling the concentrations of glucose.

Correspondence: Dr D-K Oh, Department of Bioscience and Biotechnology, Sejong University, 98 Kunja-Dong, Kwangjin-Ku, Seoul 143-747, South Korea
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Materials and methods

Microorganism and media

Torula sp. was isolated from 40% sucrose solution at the R&D Center of Bolak (Osan, Korea) [6]. The growth medium consisted of 200 g l^{-1} glucose and 10 g l^{-1} yeast extract. The production medium contained 200 g l^{-1} glucose, 10 g l^{-1} yeast extract, 10 mg l^{-1} $MnSO_4 \cdot 4H_2O$, and 2 g l^{-1} $CuSO_4 \cdot 5H_2O$ in flask cultures. Supplemental phosphate was provided to investigate the effect of phosphate. In batch and fed-batch cultures, the carbon and nitrogen sources were 400 g l^{-1} glucose and 20 g l^{-1} yeast extract, respectively. In experiments on the effect of the initial glucose concentration on erythritol production, the ratio of glucose and yeast extract (g/g) was constant at 20. In a fed-batch culture with a phosphate source, supplemental 3.3 mM phytic acid (myoinositol hexaphosphate) was provided as a phosphate source in the production medium.

Culture conditions

A single colony of *Torula* sp. was inoculated into a test tube 20 mm in diameter containing 5 ml of growth medium, and incubated at 30°C, 250 rpm for 48 h. Five milliliters of the broth was transferred into a 500-ml baffled flask containing 100 ml growth medium and cultivated at 30°C, 250 rpm for 24 h. This seed culture was then transferred into a baffled flask or a fermentor. Flask experiments were performed using 500-ml baffled flasks containing 100 ml production medium at 34°C, 250 rpm for 120 h. The initial pH of the production medium was adjusted to 5.5 and the pH of the flask culture was not controlled. Batch culture in a fermentor was performed with 5-l jar fermentors (Korea Fermentor, Incheon, Korea) containing 3 l production medium. The working volume in a fed-batch culture was increased from 2.4 to 3.0 l by continuously feeding 0.6 l of 80% glucose solution with a pump. The temperature and pH of the fermentor were controlled at 34°C and 5.5, respectively. The agitation speed was adjusted in the range of 500–850 rpm in order to maintain the dissolved

Table 1 Effect of the initial glucose concentration on fermentation parameters of *Torula* sp. in a fermentor culture: μ_{max} maximum specific growth rate; Q_P volumetric production rate of erythritol; $Y_{P/S}$ erythritol yield from glucose; t_f fermentation time

Glucose (g l^{-1})	Cell mass (g l^{-1})	Erythritol (g l^{-1})	μ_{max} (h^{-1})	Q_P (g l^{-1} h^{-1})	$Y_{P/S}$ (g g^{-1})	t_f (h)
200	18.9	82	0.036	1.03	0.41	80
250	21.1	113	0.036	1.41	0.45	80
300	22.4	160	0.034	2.19	0.53	73
350	20.4	180	0.033	1.80	0.51	100
400	18.0	193	0.032	1.43	0.41	135

oxygen concentration above 20%. The aeration rate was 0.5 vvm during fermentation.

Analytical methods

Cell dry weight was estimated using a calibration curve derived from the relationship between the absorbance at 600 nm and cell dry weight. The concentration of dissolved oxygen in the liquid phase was monitored with an Ingold polarographic electrode. Phosphate concentration was measured by the method of Murphy and Riley [9]. The concentrations of glucose and erythritol were determined by high-performance liquid chromatography coupled to a refractive index detector (Waters 410, Milford, MA, USA) a and KR100-10NH_2 Column (4.6 mm×250 mm; Kromasil, Stockholm, Sweden). The mobile phase was acetonitrile/water (80:20 v/v) and the flow rate was 1.5 ml min^{-1}.

Results and discussion

Effect of glucose concentration on erythritol production

A high initial concentration of glucose favors erythritol production by osmophilic microorganisms [5,11]. Generally, an increase in the initial glucose concentration increases the production rate and yield in a batch process if the microorganisms can tolerate a higher concentration of glucose and a higher osmotic pressure. Ishizuka *et al.* [5] increased the erythritol production rate by increasing the initial glucose concentration to 400 g l^{-1} in a culture of *Aureobasidium* sp. In erythritol production by *Trichosporon* sp., a maximum erythritol production was achieved at 220 g l^{-1} glucose, but production declined at 300 g l^{-1} glucose [11]. This might have been due to an osmotic effect on cells, or to substrate repression of glucose-metabolizing enzymes.

To evaluate the effect of the initial concentration of glucose on the fermentation parameters of *Torula* sp., the initial glucose concentration in a 5-l fermentor was varied from 200 to 400 g l^{-1} (Table 1). Increasing the initial glucose concentration increased the final erythritol concentration, but decreased the specific growth rate. The volumetric production rate of erythritol, the erythritol yield from glucose, and the final cell concentration were maximal at an initial glucose concentration of 300 g l^{-1}, and this concentration also gave the shortest culture time. The volumetric production rate of erythritol decreased at glucose concentrations below 300 g l^{-1} due to a decrease in the glucose consumption rate, whereas at glucose concentrations above 300 g l^{-1}, it decreased due to increased lag time. The optimum concentration of glucose for erythritol production was therefore determined to be 300 g l^{-1}.

A controlled glucose concentration has a better effect on erythritol production and the controlled glucose concentration was evaluated in a fermentor. Adjusting the pump speed of the feeding solution effectively controlled the glucose concentration. The concentration of the culture medium was varied within ±10 g l^{-1} during glucose feeding periods for glucose concentration settings of 150, 175, 200, 225, and 250 g l^{-1}. The volumetric production rate of erythritol was calculated by dividing the increase in erythritol concentration into the glucose-feeding period. The volumetric production rate varied substantially with the controlled glucose concentration, as shown in Figure 1. The maximal volumetric production rate was 2.49 g l^{-1} h^{-1} at a controlled glucose concentration of 225 g l^{-1}.

Increased erythritol production by controlling glucose concentration in fed-batch culture

Fed-batch culture was performed with an initial glucose concentration of 300 g l^{-1}, since the batch culture at this concentration exhibited the maximum volumetric production rate of erythritol. When the concentration of glucose fell below 225 g l^{-1}, glucose feeding was started. The volume of culture medium in the fermentor was increased from 2.4 to 3 l by continuously feeding 0.6 l of 80% glucose, and the total glucose used in the culture was 400 g l^{-1} (Figure 2). An erythritol concentration of 40 g l^{-1} was reached at 29 h in the fed-batch culture with 400 g l^{-1} glucose, whereas this concentration was not reached until 58 h in the batch culture with 400 g l^{-1} glucose (Figure 3). A cell mass of 10 g l^{-1} was seen at

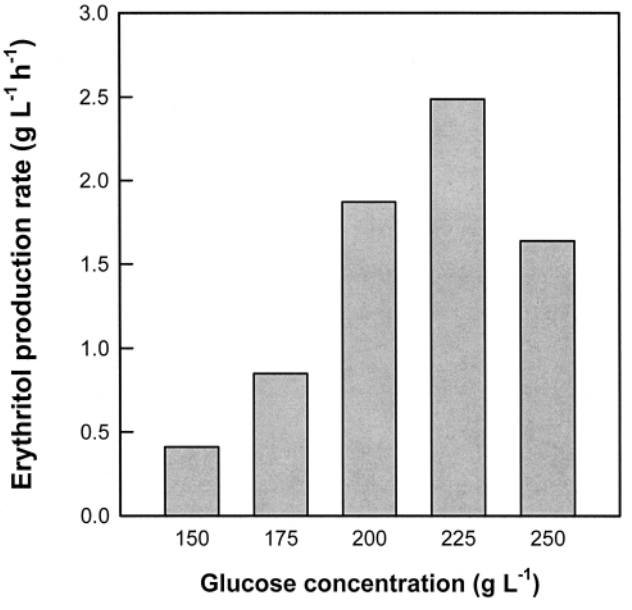

Figure 1 Effect of controlled glucose concentration on the volumetric production rate of erythritol in a fermentor.

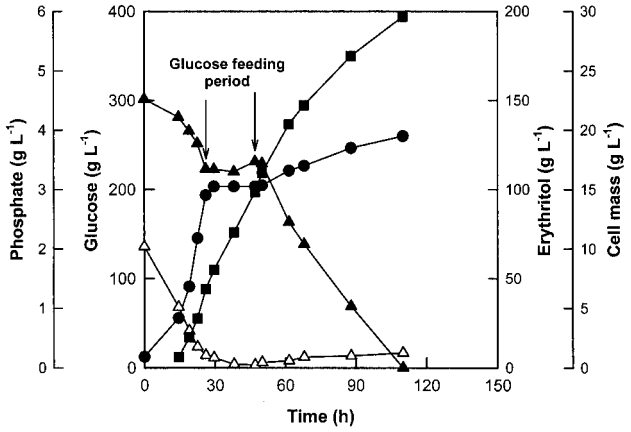

Figure 2 A fed-batch culture with glucose concentration controlled at 225 g l^{-1} and a total concentration of added glucose of 400 g l^{-1} in a fermentor. Cell mass (●), glucose (▲), phosphate (△), and erythritol (■).

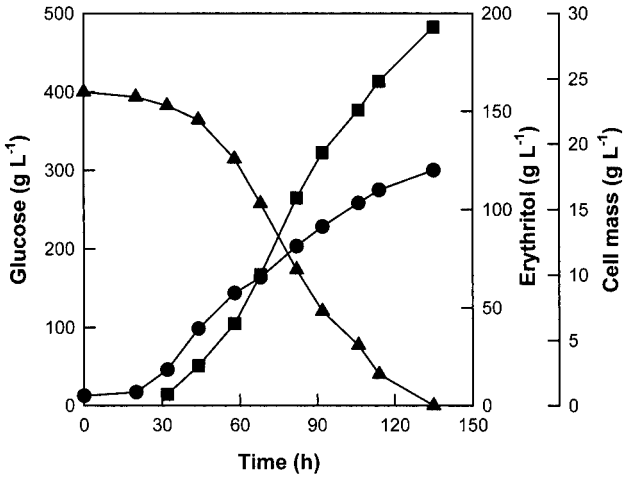

Figure 3 A batch culture with an initial 400 g l^{-1} glucose in a fermentor. Cell mass (●), glucose (▲), and erythritol (■).

20 h in the fed-batch culture, whereas this concentration was not seen until 68 h in the batch culture. The lag times of cell growth and erythritol production were therefore reduced in the fed-batch culture, and the volumetric production rate of erythritol increased. This suggests that maintaining the glucose concentration within an optimum range can increase the volumetric production rate of erythritol. However, there was a decrease in the volumetric production rate of erythritol after about 60 h in the fed-batch culture.

Phosphate is one of the most important medium components, and it affects cell physiology and metabolism in cultures of microorganisms. To investigate why the volumetric production rate decreased when the production medium did not contain a phosphate source, the phosphate concentration of the medium during the culture was determined. Culture media require phosphate in the range of 0.3–300 mM for the growth of microorganisms, and a phosphate concentration much lower than this inhibits the production of many metabolites [15]. The initial concentration of phosphate from yeast extract was 2 g l^{-1} (14.7 mM) and the minimum concentration was 0.04 g l^{-1} (0.3 mM). This phosphate concentration was thought insufficient, and a phosphate source must therefore be included for effective erythritol production.

Increased erythritol production by controlling glucose concentration in fed-batch cultures with phytic acid

In order to select the optimum phosphate source, the erythritol concentration was determined after 120 h of cultivation in baffled flasks using several phosphate sources in the production medium (Table 2). Equivalent amounts of phosphate were calculated as the PO_4^{-3} form. The supplemented concentration of compounds containing one phosphate group, such as KH_2PO_4, NaH_2PO_4, and $NH_4H_2PO_4$, was 13.2 mM, and that using phytic acid (mono-inositol hexaphosphate) was 2.2 mM. Phytic acid has six covalently bonded phosphate groups and accounts for up to 85% of the total phosphorus in many cereals and legumes. There are 12 replaceable protons in the phytic acid molecule, giving it the ability to complex with multivalent cations and positively charged proteins. Phosphate supplementation stimulated cell growth and erythritol production, and of the phosphate sources tested, phytic acid was the best. The increased erythritol production with supplemental phytic acid may be due to slow release of phosphate. The effect of phytic acid addition on cell growth and erythritol production was dependent on concentration,

Table 2 Effect of phosphate source on cell growth and erythritol production in a flask culture using the production medium

Phosphate source	Concentration (g l^{-1}, mM)	Cell mass (g l^{-1})	Erythritol (g l^{-1})
Control	0.0/0.0	14.4	61.0
KH_2PO_4	1.80/13.2	16.2	66.3
NaH_2PO_4	1.58/13.2	17.2	65.8
$NH_4H_2PO_4$	1.52/13.2	15.3	76.4
Phytic acid	2.00/2.2	18.8	90.0

The same amount of phosphate was used when calculated as the PO_4^{-3} form. The concentrations of cells and erythritol were determined after 120 h of cultivation.

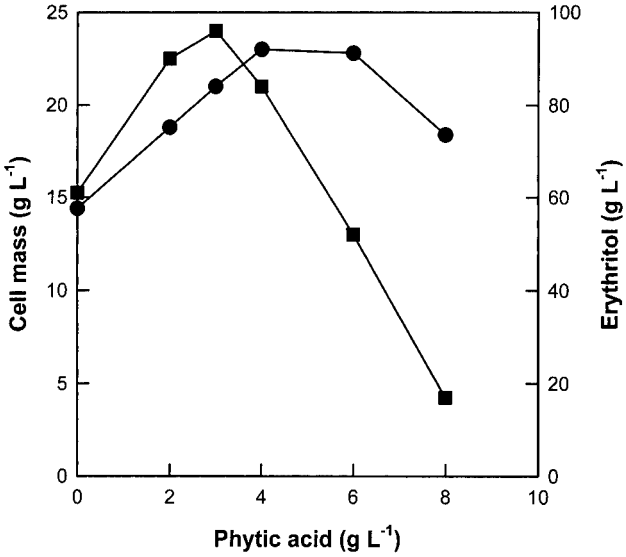

Figure 4 The effect of phytic acid concentration on cell growth and erythritol production in a flask culture using the production medium. Cell mass (●) and erythritol (■).

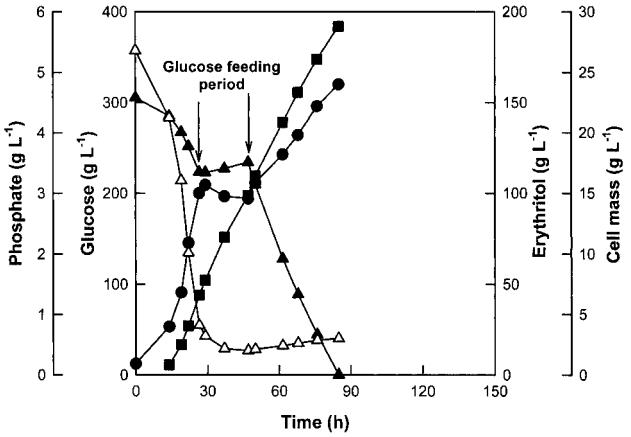

Figure 5 A fed-batch culture with a controlled glucose concentration of 225 g l^{-1} in medium containing 3 g l^{-1} phytic acid and a total concentration of added glucose of 400 g l^{-1} in a fermentor. Cell mass (●), glucose (▲), phosphate (△), and erythritol (■).

as shown in Figure 4. Erythritol production was maximal at 3 g l^{-1} (3.3 mM) phytic acid.

A fed-batch culture with a controlled glucose concentration of 225 g l^{-1} was performed with medium containing 3 g l^{-1} phytic acid. As shown in Figure 5, phosphate was produced in the fed-batch culture from yeast extract and phytic acid. The initial phosphate concentration was 5.4 g l^{-1} (39.7 mM) and the minimum concentration was 0.4 g l^{-1} (2.9 mM), almost 10 times that of a fed-batch culture without a phosphate source. This phosphate concentration was thought to be sufficient for effective erythritol production. The volumetric production rate of erythritol in the fed-batch culture containing phytic acid did not decrease even after about 60 h due to an increase of cell mass. Generally, phosphate stimulates cell growth [15], and this

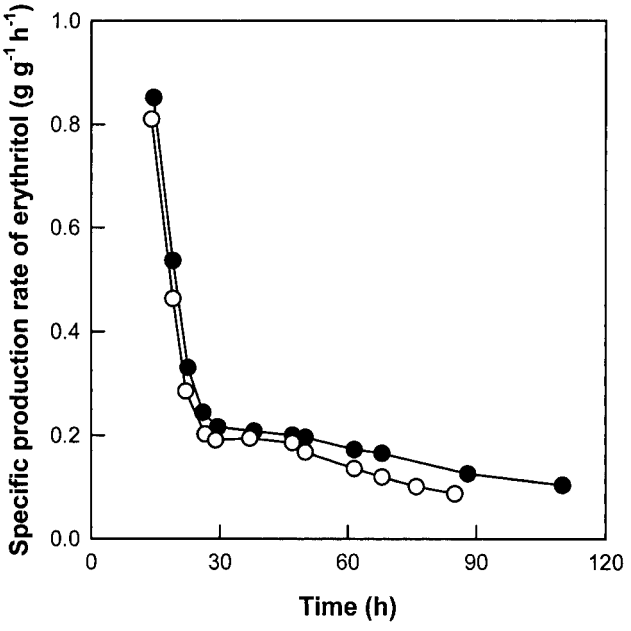

Figure 6 Specific production rates of fed-batch cultures during erythritol production by *Torula* sp. Specific production rate of a fed-batch culture with (○) and without (●) a phosphate source.

Table 3 Comparison of fermentation parameters for batch and fed-batch cultures with a total of 400 g l^{-1} glucose: μ_{max} maximum specific growth rate; Q_P volumetric production rate of erythritol; $Y_{P/S}$ erythritol yield from glucose; t_f fermentation time

Culture	Cell mass (g l^{-1})	Erythritol (g l^{-1})	μ_{max} (h^{-1})	Q_P (g l^{-1} h^{-1})	$Y_{P/S}$ (g g^{-1})	t_f (h)
Batch	18.0	193	0.032	1.43	0.48	135
Fed-batch	19.8	197	0.040	1.79	0.49	110
Fed-batch with phytic acid	24.1	192	0.042	2.26	0.48	85

suggests that phytic acid increased cell mass and reduced culture time, resulting in an increase in the volumetric production rate of erythritol.

In order to investigate the reason for the increase in the volumetric production rate of erythritol in the fed-batch culture with phosphate, the specific production rate of erythritol (Figure 6) was calculated from data shown in Figures 3 and 5. The specific production rates in the fed-batch culture with phosphate were approximately the same as those without phosphate. Since the specific production rate multiplied by the cell concentration gives the volumetric production rate, the increase in the volumetric production rate of erythritol in the fed-batch culture with phosphate was not due to the specific production rate, but to the increased cell concentration. Supplemental phosphate in the fed-batch culture prevented phosphate deficiency and increased cell mass, resulting in an increase in the volumetric production rate.

The fermentation parameters for batch and fed-batch cultures with a total of 400 g l^{-1} glucose were compared, as shown in Table 3. The volumetric production rate of erythritol in the fed-batch culture with a phosphate source was 58% higher than that obtained in the batch culture. The fed-batch culture with controlled glucose concentration containing phosphate increased the volumetric production rate of erythritol.

Erythritol production from glucose by various yeasts is summarized in Table 4. *Aureobasidum* sp. grown on a medium containing 400 g l^{-1} glucose produced 175 g l^{-1} erythritol over a period of 96 h. This corresponds to a volumetric production rate of 1.82 g l^{-1} h^{-1} [5], the highest previously reported volumetric production rate of erythritol. However, a culture of *Torula* sp. in our study exhibited the highest erythritol yield from glucose and highest volumetric production rate of erythritol yet reported. The respective volumetric production rate and yield of erythritol with *Torula* sp. in a fed-batch culture were

Table 4 Erythritol productions from various microorganisms: Q_P volumetric production rate of erythritol; $Y_{P/S}$ erythritol yield from glucose

Microorganism	Glucose (g l^{-1})	Erythritol (g l^{-1})	Q_P (g l^{-1} h^{-1})	$Y_{P/S}$ (g g^{-1})	Reference
Aureobasidium sp.	400	175	1.82	0.44	[5]
Moniliella tomentosa var. *pollinis*	357	133	0.79	0.37	[4]
Trichosporon sp.	300	138	1.23	0.46	[11]
Fed-batch culture	333	150	1.50	0.45	
Torula sp.	300	160	2.19	0.48	This study
Fed-batch culture	400	192	2.26	0.48	

approximately 150% and 10% higher than values previously attained with *Trichosporon* sp. in a fed-batch culture [11]. In this study, using *Torula* sp., the volumetric production rate of erythritol was increased by controlling the concentrations of glucose in a medium containing phosphate sources. These results should contribute to better industrial production of erythritol by microbiologic processes.

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