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Optimization of Culture Conditions for Erythritol Production by *Torula* sp.

KIM, KYUNG-AH, BONG-SOO NOH, JUNG-KUL LEE[1], SANG-YONG KIM[1], YONG-CHEOL PARK[2], AND DEOK-KUN OH[3]*

Department of Food and Microbial Technology, Seoul Women's University, Seoul 139-774, Korea
[1]Dong Cheon Consulting Co., Kyunggi-Do 445-930, Korea
[2]Department Research Center for New Bio-materials in Agriculture, Seoul National University, Suwon 441-744, Korea
[3]Department of Bioscience and Biotechnology, Sejong University, Seoul 143-747, Korea

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Abstract The medium for erythritol production by *Torula* sp. in a 500-ml baffled flask was optimized to be 300 g/l sucrose, 10 g/l yeast extract, 3 g/l KH_2PO_4, and 10 mg/l $CuSO_4 \cdot 5H_2O$ at 34°C with initial pH of 5.5. Using this optimal medium, erythritol of 166 g/l was obtained after 140 h of cultivation, corresponding to 55.3% of the erythritol yield from sucrose with a productivity of 1.11 g/l/h. Optimal concentrations of carbon and nitrogen sources in a fermentor were higher than that in a flask due to the higher oxygen supply of the fermentor. Employing the medium containing 300 g/l or 400 g/l sucrose for the determination of optimal C/N ratio, the C/N ratio was found to be more important than the nitrogen concentration for effective erythritol production. The optimal ratio of yeast extract to sucrose (g/g) was 20. The yield and productivity of erythritol were maximal in the medium containing 400 g/l sucrose and 20 g/l yeast extract. When dissolved oxygen in the culture was increased, the cell mass increased but the erythritol production was maximal in the range of 5 to 10% of dissolved oxygen. Under the optimal culture condition of the fermentor, a final erythritol concentration of 200 g/l was obtained after 120 h with a yield of 50% and the productivity was 1.67 g/l/h. The yield was the highest among erythritol-producing microorganisms

Key words: *Torula* sp., erythritol, sucrose, yeast extract, optimization of culture conditions

Erythritol is a four-carbon polyol with properties similar to other polyols presently used as food ingredients such as xylitol, sorbitol, mannitol, maltitol, lactitol, and isomalt [2]. It is a naturally occurring substance and is widely distributed in nature [3]. Erythritol is a metabolite or storage compound for seaweed and mushrooms. Fruits like melons, grapes, and pears contain erythritol. It occurs frequently in fermented food including wines and beers, and processed vegetables such as soy sauce and oriental miso bean paste [16, 17, 18].

Erythritol has a sweetness of 60 to 70% of sucrose in a 10% solution. It has a substantially high negative heat when dissolved in solution, providing a strong cooling effect [3]. Erythritol can also be safely used in foods to make them tooth-friendly. This property is due to the inability of dental caries developing bacteria to use erythritol as a fermentation substrate. As a small molecule, erythritol has strong colligative properties such as strong freezing point depression, boiling point elevation, and high osmotic pressure. With its low hygroscopicity and viscosity in solution, it is very useful to reduce and to control the water activity of foodstuff [4].

Erythritol can be produced by microbial methods using osmophilic yeasts, especially species of the genus *Torulopsis* [13] such as *T. magnoliae*, *T. veratilis*, and *T. candida*; *Endomycopsis chodati* [13]; *Hansenula supelliculsa*; *Pichia miso* [13]; *Monilliella tomentosa* var. *pollinis* [5]; *Trigonopsis variabilis* [10]; *Trichosporonoides* [1]; *Candida zeylanoides* [6]; and *Aureobasidium* sp. [7]. *Monilliella tomentosa* var. *pollinis* produced erythritol on a medium containing 35.7% glucose with a yield of 45.6% [5]. Erythritol production using this strain could not be applied to an industrial scale due to by-products such as glycerol and ribitol. Industrial production of erythritol has been performed using a mutant of *Aureobasidium* [7]. The mutant produced erythritol with a 1.82 g/l/h volumetric productivity and 43.8% yield in a medium containing 40% glucose. This is the highest reported erythritol productivity among the erythritol-producing microorganisms.

It has not been previously reported that *Torula* sp. produced erythritol. The *Torula* sp. used in this study was isolated from a 40% sucrose solution [9]. Using this strain for erythritol production, the optimization of culture

*Corresponding author
Phone: 82-2-3408-3643; Fax: 82-2-3408-3569,
E-mail: deokkun@sejong.ac.kr

conditions such as medium components and environmental conditions were determined.

MATERIALS AND METHODS

Microorganism and Media

Torula sp. was isolated from a 40% sucrose solution at the R&D Center of Bolak Co. (Osan, Korea) [9]. The growth medium consisted of 200 g/l glucose and 10 g/l yeast extract. The production medium was modified on the basis of growth medium.

Culture Conditions

A single colony of *Torula* sp. was inoculated to a 20-mm diameter test tube containing 5 ml of growth medium and was 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 was 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 a 500-ml baffled flask containing 100 ml production medium at 34°C, 250 rpm. Culture time was 120 h in the medium containing a 200 g/l carbon source and 140 h in the medium containing 300 g/l sucrose. The initial pH of the production medium was adjusted to 5.5. Fermentor experiments were performed with 5-l jar fermentors (Korea Fermentor Co., Incheon, Korea) containing 3 l production medium. The temperature and pH of the fermentor were controlled at 34°C and 5.5, respectively. Agitation speed was adjusted in the range of 500 to 850 rpm for the 24 h cultivation in order to maintain a dissolved oxygen concentration at above 20%. After 24 h, it was fixed to 600 rpm. The aeration rate was 0.5 vvm during fermentation.

Analytical Methods

The dry cell weight was estimated by using a calibration curve derived from the relationship between absorbance at 600 nm and dry cell weight. Glucose concentration was measured by a glucose oxidase kit (Young-Dong Pharmaceutical Co., Seoul, Korea). Erythritol concentration was determined by using high performance liquid chromatography (Waters 510, Massachusetts, U.S.A.) coupled to a refractive index detector (Waters 410, Massachusetts, U.S.A.) and an NH_2 column (Kromasil, Stockholm, Sweden). The mobile phase was acetonitrile/water (80:20 v/v) at the flow rate of 1.5 ml/min [17].

RESULTS AND DISCUSSION

Characteristics of Isolated Strain

On the basis of results obtained with the isolated strain, we characterized this organism as a *Torula* sp. (Table 1).

Effect of Carbon Source on Erythritol Production in a Baffled Flask

In order to select the optimal carbon source, the erythritol produced from various carbon sources was determined after 120 h of cultivation in baffled flasks containing medium composed of 200 g/l carbon source and 10 g/l yeast extract (Table 2). The carbon sources used were monosaccharides such as glucose, galactose, mannose, and sorbose; and disaccharides such as sucrose and lactose. Galactose and mannose exhibited a high cell growth but low erythritol production. Lactose was not consumed by *Torula* sp. and sorbose inhibited cell growth and erythritol production. Among the various carbon sources used, sucrose was the best carbon source for erythritol production.

The effect of sucrose concentration on cell growth and erythritol production was investigated in the medium containing 10 g/l yeast extract. As shown in Fig. 1, cell growth, erythritol production, and erythritol yield were maximal at a 300 g/l initial sucrose concentration. The decrease of these values at a high concentration of sucrose may be due to the limitation of dissolved oxygen in the baffled flask.

Effect of Nitrogen Source on Erythritol Production in a Baffled Flask

The effect of nitrogen source on cell growth and erythritol production was investigated in the medium containing 300 g/l sucrose and nitrogen source. Cells and erythritol produced from 300 g/l sucrose were analyzed after 140 h of cultivation. The concentration of nitrogen source was adjusted to the same content of nitrogen, supplied by Difco, using the Kjeldahl method. The nitrogen content of 10 g/l yeast extract was the same as that of 6.94 g/l peptone, 8.01 g/l tryptone, and 10 g/l casamino acid. As shown in Table 3, yeast extract was the best nitrogen source for cell growth and erythritol production among the nitrogenous compounds tested. The effect of yeast extract concentration on cell growth and erythritol production in the medium containing 300 g/l sucrose was studied to determine its optimal concentration in a baffled flask (Fig. 2). Yeast extract concentrations varied from 5 to 25 g/l. The cell mass was maximal at 20 g/l yeast extract whereas the erythritol production was maximal at 10 g/l yeast extract. Therefore, the optimum concentration of yeast extract for erythritol production was determined to be 10 g/l.

Effect of Phosphate Source on Erythritol Production in a Baffled Flask

The effect of phosphate concentration on cell growth and erythritol production was investigated at various concentrations of KH_2PO_4 in the medium containing 300 g/l sucrose and 10 g/l yeast extract. As shown in Fig. 3, cell mass and erythritol production were maximal at 5 g/l KH_2PO_4 and therefore, concluded to be the optimum concentration of the phosphate source.

Table 1. Microbial characteristics of the erythritol-producing strain.

Description:						
Cream colonies: vegetative reproduction by budding; not filamentous; no sexual reproduction.						
Fermentation:						
	D-Glucose	+	D-Galactose	-	D-Mannose	+
	D-Fructose	+	Maltose	-	Sucrose	+
	Trehalose	-	Melibiose	-	Lactose	-
	Cellobiose	-	Melezitose	-	Raffinose	-
	Starch	-	Inulin	-	D-Xylose	-
Growth:						
	D-Glucose	+	D-Galactose	+	D-Mannose	+
	D-Fructose	+	L-Sorbose	+	D-Glucosamine	
	D-Ribose	+	D-Xylose	+	D-Arabinose	+
	L-Rhamnose	+	Maltose	-	Sucrose	+
	Palatinose	+	Trehalulose	+	Trehalose	-
	Melibiose	-	Salicin	+	Arbutin	+
	Lactose	-	Cellobiose	-	Melezitose	-
	Raffinose	-	Starch	-	Inulin	-
	Glycerol	+	Erythritol	-	Ribitol	+
	Xylitol	+	L-Arabinitol	-	D-Glucitol	+
	D-Mannitol	+	Galactitol	-	*myo*-Inositol	-
	D-Gluconate	+	D-Glucuronate	-	D-Galacturonate	-
	DL-Lactate	-	Succinate	+	Citrate	+
	Methanol	-	Ethanol	-	Propanediol	-
	Nitrate	-	Ethylamine	+	L-Lysine	+
	Cadaverine	+	Creatine	-	Creatinine	-
	Glucosamine	-	Imidazole	-	w/o vitamins	-
	25°C	+	37°C	+	40°C	-
	0.01% Cycloheximide	+	0.1% Cycloheximide	-		
	1.0% Acetic acid	-	50% D-Glucose	+		
Additional characteristics:						
	Starch formation	-	Acetic acid production	-		
	Urea hydrolysis	-	Diazonium Blue B reaction	-		

Table 2. Erythritol productions from various carbon sources by *Torula* sp. in baffled flasks.

Carbon sources (200 g/l)	Cell mass (g/l)	Erythritol (g/l)	Yield (%)	Productivity (g/l/h)
Glucose	9.5	40.2	20.1	0.34
Galactose	15.8	28.3	14.2	0.24
Mannose	16.4	32.7	16.4	0.27
Fructose	11.6	21.4	10.7	0.18
Sorbose	4.5	5.2	2.6	0.04
Sucrose	6.8	53.8	26.9	0.45
Lactose	-	-	-	-

Effect of Inorganic Salt on Erythritol Production in a Baffled Flask

The experiments to determine the optimal concentrations of inorganic salts such as magnesium, copper, iron, zinc, and calcium for erythritol production were performed in the medium containing 300 g/l sucrose, 10 g/l yeast extract,

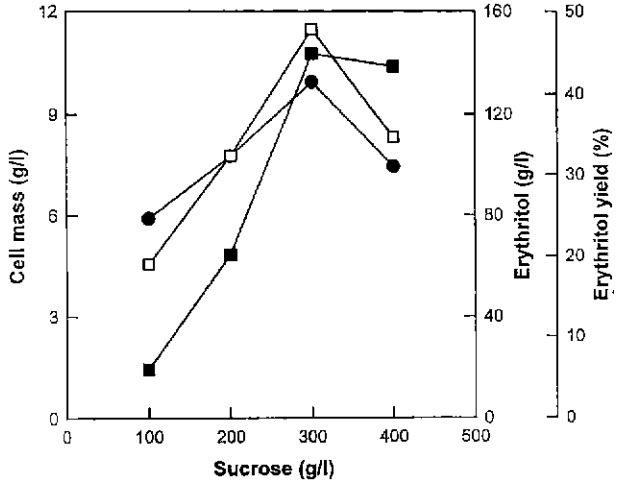

Fig. 1. Effect of sucrose concentration on cell growth and erythritol production.
—●—, cell mass; —■—, erythritol concentration; —□—, erythritol yield from sucrose.

Table 3. Erythritol productions from various nitrogen sources by *Torula* sp. in baffled flasks.

Nitrogen source	Concentration (g/l)	Cell mass (g/l)	Erythritol (g/l)	Yield (%)	Productivity (g/l/h)
Casamino acid	10.0	4.9	68.2	22.7	0.49
Peptone	6.9	3.9	28.8	9.6	0.21
Tryptone	8.0	7.2	80.2	26.7	0.57
Yeast extract	10.0	10.4	143.4	47.8	1.02

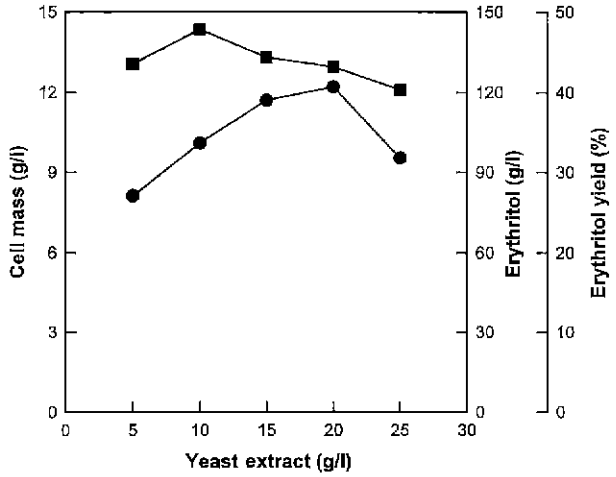

Fig. 2. Effect of yeast extract concentration on cell growth and erythritol production.
—●—, cell mass; —■—, erythritol concentration, erythritol yield from sucrose

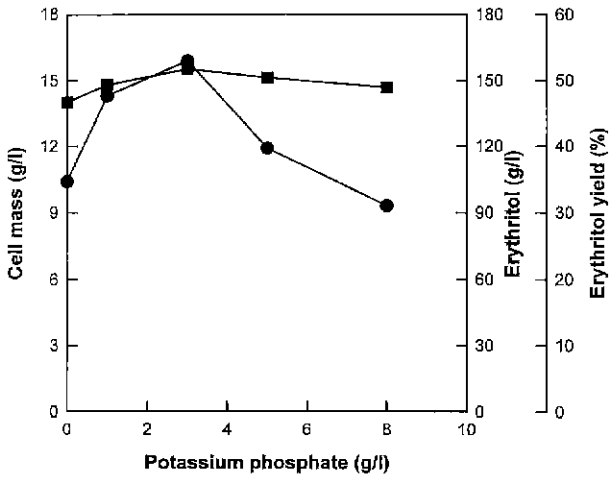

Fig. 3. Effect of potassium phosphate concentration on cell growth and erythritol production.
—●—, cell mass; —■—, erythritol concentration, erythritol yield from sucrose.

5 g/l KH_2PO_4, and inorganic salt. Erythritol concentrations were approximately constant in spite of different concentrations of the salts such as $MgSO_4 \cdot 7H_2O$ (0.2–1.0 g/l), $FeSO_4 \cdot 7H_2O$ (10–250 mg/l), and $ZnSO_4 \cdot 7H_2O$ (10–250 mg/l). The addition of $CaCl_2 \cdot 7H_2O$ in the range of 10 to 250 mg/l resulted in the decrease of erythritol production (data was

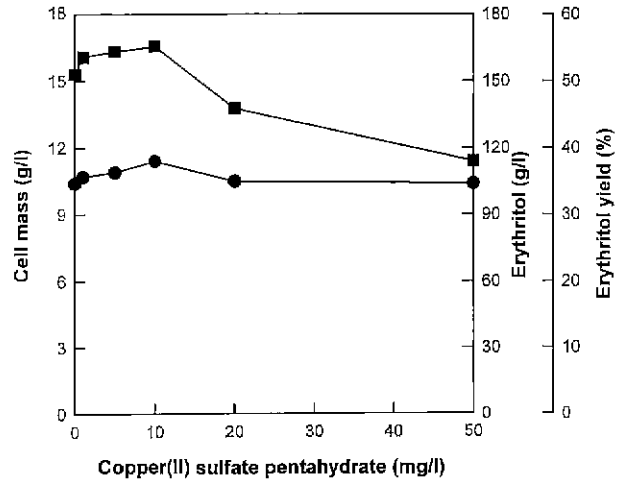

Fig. 4. Effect of copper (II) sulfate pentahydrate concentration on cell growth and erythritol production.
—●—, cell mass, —■—, erythritol concentration, erythritol yield from sucrose

not shown). The effect of $CuSO_4 \cdot 5H_2O$ concentration on the erythritol concentration, however, was significant as shown in Fig. 4. Cell mass and erythritol production were maximal at 10 mg/l $CuSO_4 \cdot 5H_2O$ so this was determined ot be the optimum concentration. Throughout the experiments, the optimal medium in a baffled flask was therefore decided to contain 300 g/l sucrose, 10 g/l yeast extract, 5 g/l KH_2PO_4, and 10 mg/l $CuSO_4 \cdot 5H_2O$.

Effect of C/N Ratio on Erythritol Production in a Fermentor

The optimal concentrations of carbon and nitrogen sources for effective erythritol production in a fermentor have been considered to be higher than those in a flask due to higher oxygen supply in the fermentor. In order to optimize the C/N ratio, the changes of cell mass and erythritol production by varying yeast extract concentration were studied in the media containing 300 g/l sucrose and 400 g/l sucrose (Fig. 5). It was of interest to note that the C/N ratio was more important than nitrogen concentration for the increase of cell growth and erythritol production. The optimal ratio of yeast extract to sucrose (g/g) was found to be 20, such as 300 g/l sucrose to 15 g/l yeast extract or 400 g/l sucrose to 20 g/l yeast extract. The C/N ratio is known to be an important parameter for the production of metabolites such as poly(3-hyroxyalkanoates) and carotenoids [8, 12].

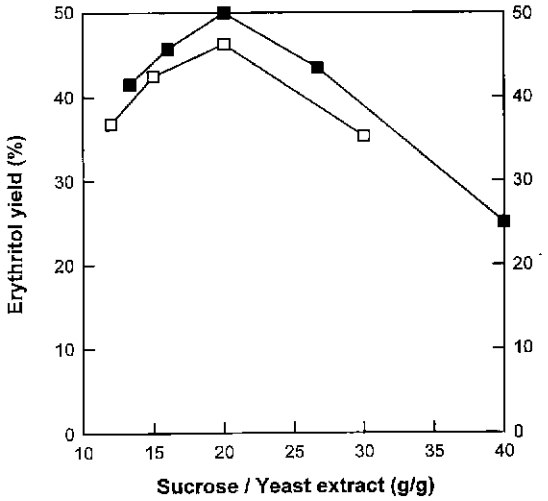

Fig. 5. Effect of the ratio of yeast extract to sucrose on cell growth and erythritol production.
—■—, erythritol concentration in 300 g/l sucrose medium; —□—, erythritol concentration in 400 g/l sucrose medium

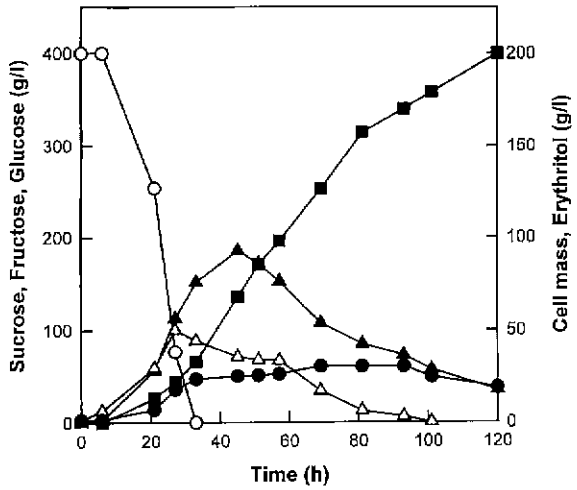

Fig. 6. Erythritol production by *Torula* sp. in a medium containing 400 g/l sucrose.
—●—, cell mass; —■—, erythritol; —○—, sucrose consumption —▲—, fructose concentration; —△—, glucose concentration.

Table 4. Effect of sucrose concentration on erythritol production by *Torula* sp. at the optimal C/N ratio in a 5-l fermentor.

Sucrose (g/l)	300	400	500
Yeast extract (g/l)	15	20	25
Cell mass (g/l)	25.6	30.4	43.3
Erythritol (g/l)	139.0	200.0	234.2
Yield (%)	46.3	50.0	46.8
Productivity (g/l/h)	1.26	1.67	1.63
Residual sugar (g/l)	-	[a]F35.0	[a]F73.8, G72.6
Fermentation time (h)	110	120	144

[a]F=Fructose, G=Glucose.

Table 5. Effect of dissolved oxygen on erythritol production by *Torula* sp. in a 5-l fermentor.

Agitation speed (rpm)	400[a]	500[a]	600[a]	500–850
Dissolved oxygen (%)	1.5–2.0	2.0–5.0	5.0–10	20±1
Cell mass (g/l)	14.5	16.9	30.4	43.3
Erythritol (g/l)	157.7	185.3	200.0	150.0
Yield (%)	39.4	46.3	50.0	37.5
Productivity (g/l·h)	1.13	1.43	1.67	1.50
Residual sugar (g/l)	[a]F109.0	[a]F68.0	[a]F35.0	-
Fermentation time (h)	140	130	120	100

[a]F=Fructose.

The effect of sucrose concentration on cell growth and erythritol production was studied in the medium with the optimal C/N ratio (Table 4). The yield and productivity of erythritol were maximal at 400 g/l sucrose and 20 g/l yeast extract. However, the final erythritol concentration was found to be maximal at 500 g/l sucrose.

Effect of Dissolved Oxygen on Erythritol Production in a Fermentor

The dissolved oxygen during erythritol fermentation is an important factor because it controls the reduction reaction in converting erythrose to erythritol [15]. The effect of dissolved oxygen on erythritol production, however, has not been previously reported. Experiments were performed at various agitation speeds in 400 g/l sucrose medium to investigate the effect of dissolved oxygen on cell growth and erythritol production (Table 5). Agitation speed was adjusted in the range of 500 to 850 rpm for 24 h of cultivation in order to maintain the dissolved oxygen at above 20%. After 24 h, it was fixed to 400, 500, and 600 rpm, respectively. In an another experiment, agitation speed was controlled in the range of 500 to 850 rpm to maintain the dissolved oxygen of 20% during fermentation. When the dissolved oxygen of the culture increased, the cell mass increased and erythritol production was maximal at 600 rpm. These results suggest that the level of dissolved oxygen during fermentation must be controlled in the range of 5 to 10%.

Results from typical batch fermentations with an initial sucrose concentration of 400 g/l are illustrated in Fig. 6. Sucrose and glucose formed from sucrose were completely consumed after 33 h and 100 h, respectively, whereas fructose produced from sucrose still remained unutilized after 120 h of cultivation. Erythritol production and fructose consumption were inhibited after 120 h due to the depletion of glucose. The concentrations of glucose and fructose were maximal at 27 h and 45 h of cultivation, respectively. Glucose and fructose were simultaneously consumed. A final erythritol concentration of 200 g/l was obtained after 120 h of cultivation, where the yield was 50% with 1.67 g/l/h productivity.

Table 6. Erythritol productions from various microorganisms.

Microorganism [Reference]	Sugar (g/l)	Erythritol (g/l)	Yield (%)	Productivity (g/l/h)
Aureobasidium sp. [7]	Glucose 400	175	43.8	1.82
Candida sp. [11]	Sucrose 300	80.2	26.7	0.47
Moniliella tomentosa var. *pollinis* [5]	Glucose 357	133	37.3	0.79
Trichospronoides sp.[1]	Sucrose 100	37.4	37.4	0.26
Trichosporon sp. [14]	Glucose 400	188	47.0	1.18
Torula sp. [This study]	Sucrose 400	200	50.0	1.58
	Sucrose 300	166	55.3	1.19

Aureobasidium sp. grown on a medium containing 400 g/l glucose produced 175 g/l of erythritol over a period of 96 h [7]. This erythritol productivity was the highest reported. The yield of erythritol from sugar, however, exhibited the highest value at culture with *Torula* sp. in this study among the reported microorganisms (Table 6). The productivity and yield of erythritol with *Torula* sp. from 300 g/l sucrose were 2.5 times and 29%, respectively, higher than those previously attained with *Candida* sp. [11].

As compared with previous reports, the erythritol production by *Torula* sp. in this study is more superior in several aspects. First, this is the first observation that *Torula* sp. produces erythritol. Second, the highest yield of erythritol was obtained among erythritol-producing microorganisms. Third, this strain did not produce by-products such as glycerol and ribitol, resulting in potential applications to industrial scale. These results will contribute to better industrial production of erythritol by microbiological processes.

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