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Optimization of erythritol production by *Candida magnoliae* in fed-batch culture

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A two-stage fed-batch process was designed to enhance erythritol productivity by the mutant strain of *Candida magnoliae*. The first stage (or growth stage) was performed in the fed-batch mode where the growth medium was fed when the pH of the culture broth dropped below 4.5. The second stage (or production stage) was started with addition of glucose powder into the culture broth when the cell mass reached about 75 g dry cell weight l^{-1}. When the initial glucose concentration was adjusted to 400 g l^{-1} in the production stage, 2.8 g l^{-1} h^{-1} of overall erythritol productivity and 41% of erythritol conversion yield were achieved, which represented a fivefold increase in erythritol productivity compared with the simple batch fermentation process. A high glucose concentration in the production phase resulted in formation of organic acids including citrate and butyrate. An increase in dissolved oxygen level caused formation of gluconic acid instead of citric acid. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 100–103.

Keywords: *Candida magnoliae*; erythritol; fed-batch culture

Introduction

Erythritol is a non-cariogenic [5], non-caloric (0.3 kcal g^{-1}) sweetener [13] and safe for diabetics [1]. The four-carbon sugar alcohol has about 70–80% of the sweetness of sucrose and is found in fruits, mushrooms and some fermented foods.

Many research efforts have focused on the optimization of culture conditions in batch [3,4,7–10,14] and fed-batch culture processes [11]. Ishizuka *et al.* [4] isolated a strain of *Aureobasidium* sp. SN-124A and induced mutations using UV irradiation and nitrosoguanidine (NTG) treatment. The selected mutant converted glucose to erythritol with 47.6% yield. Park *et al.* [11] reported 1.86 g l^{-1} h^{-1} erythritol productivity and 45% overall erythritol conversion yield with an osmophilic yeast, *Trichosporon* sp., in a repeated fed-batch culture.

A fed-batch culture process has many advantages for improving the productivity since optimal conditions for cell growth and product formation are usually different. Two-stage fermentation processes have been designed and evaluated for the production of foreign proteins. For example, Chang *et al.* [2] optimized the production of rice α-amylase by *Yarrowia lipolytica* SMY2 ATCC 201847 in a cyclic fed-batch culture. When the volume in the first stage reached a preset value, a portion of culture broth was transferred to the second stage where a desired metabolite was produced. The remaining cells in the growth stage were then fed with fresh growth medium to resume cell growth. Park *et al.* [12] optimized and compared the performance of recombinant *Escherichia coli* containing the cloned *trp* promoter between simple batch and fed-batch fermentation processes. Kim *et al.* [6] designed a two-substrate fermentation process to increase xylitol conversion yield and volumetric productivity. Glucose was used initially for cell growth followed by conversion of xylose to xylitol without cell growth and by-product formation after complete depletion of glucose.

The objective of this research was to design a fed-batch culture process to enhance erythritol productivity by an osmophilic mutant of *Candida magnoliae*. The feeding strategy of glucose in the production stage was optimized and the effects of dissolved oxygen and organic acids on erythritol production were also analyzed.

Materials and methods

Microorganism and cultivation

An osmophilic yeast used for erythritol production was a mutant strain of *C. magnoliae* [14]. The initial culture medium for the first stage was composed of 10 g l^{-1} glucose (Sigma Chemical Co., St. Louis, MO, USA) and 6.7 g l^{-1} yeast extract (Difco Laboratories, Detroit, MI, USA). The growth medium in the first stage consisted of 316 g l^{-1} glucose and 212 g l^{-1} yeast extract. It was automatically fed whenever pH of the culture broth dropped below 4.5. The second stage was started with the addition of glucose powder into the culture broth when the cell mass reached 75 g l^{-1}. Fermentations were done at 28°C in a 3.3-l jar fermentor (NBS, New Brunswick, NJ, USA). Agitation speed and aeration rate were controlled to maintain a dissolved oxygen level above 20%. Inoculum of the mutant *C. magnoliae* was prepared by growing the strain in 250-ml cornical flasks containing 50 ml culture medium composed of 20 g l^{-1} glucose, 10 g l^{-1} yeast extract and 10 g l^{-1} peptone.

Analytical methods

Off-line measurements of dry cell weight (DCW) were conducted with a spectrophotometer (Shimadzu, Tokyo, Japan) at 620 nm wavelength. DCW was estimated by a predetermined conversion factor 0.25 g-DCW l^{-1} Abs^{-1}.

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Analysis of polyols and glucose in the culture broth was performed by using HPLC unit equipped with the NH_2 column (Waters, Milford, MA, USA) and a reflective index (RI) detector (Shisheido, Tokyo, Japan). The mobile phase was acetonitrile–water and its flow rate was 1.4 ml min^{-1}.

Concentrations of citric, butyric and gluconic acids in the culture broth were measured using the same HPLC unit equipped with an Aminex HPX-87H column (Bio-rad, Hercules, CA, USA) and a UV detector at 210 nm. The mobile phase was 5 mM H_2SO_4 with a 0.6 ml min^{-1} flow rate.

Results and discussion

Erythritol production in fed-batch culture

Fed-batch fermentations were employed to produce erythritol by C. *magnoliae*, the first stage for cell growth followed by an erythritol production phase. Feeding of growth medium was automatically carried out whenever acidity of the culture broth dropped below pH 4.5. Cells grew exponentially and reached 72 g-DCW l^{-1} after 27 h (Figure 1). Glucose concentrations in the culture broth were maintained between 2 and 20 g l^{-1}, which were determined to be optimum concentrations for cell growth

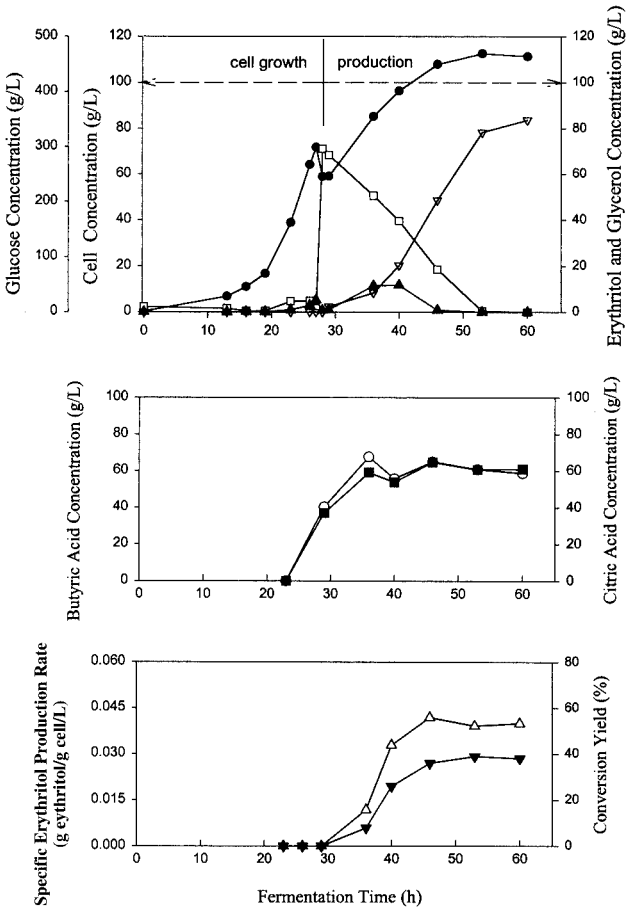

Figure 1 Profiles with 300 g l^{-1} of initial glucose concentration in the second stage. *C. magnoliae* was grown at pH 7.0 and 28°C in a 3.3-l jar fermentor. (□) Glucose; (●) cell mass; (▽) erythritol; (▲) glycerol; (○) citric acid; (■) butyric acid; (△) specific erythritol production rate; (▼) erythritol conversion yield.

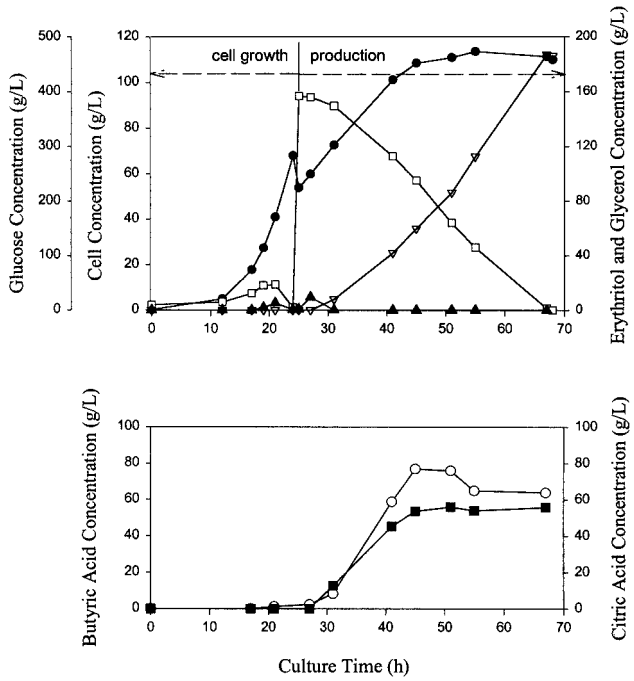

Figure 2 Trajectories of fed-batch culture of *C. magnoliae* with 400 g l^{-1} of initial glucose concentration in the second stage. (□) Glucose; (●) cell mass; (▽) erythritol; (▲) glycerol; (○) citric acid; (■) butyric acid.

(data not shown). Little erythritol was produced and 4 g l^{-1} of glycerol was formed by the end of the growth phase. The acidity of the culture broth decreased to about pH 4.2 when the first stage was finished.

The production phase was initiated by adding glucose powder to keep glucose concentrations around 300 g l^{-1}. Glucose was consumed rapidly and exhausted after 54 h. Erythritol concentrations increased at a rate of 4.1 g l^{-1} h^{-1} after a short lag period and reached 84 g l^{-1} in 60 h. Glycerol was produced in proportion to cell growth in the early stage of the production phase but was consumed later as a carbon source. Cell concentrations gradually increased to 112 g l^{-1} by the end of the production stage. Organic acids such as citric acid and butyric acid were rapidly formed in parallel with cell growth in the early stage of the production phase (Figure 1), which caused a drop of culture pH to below 3.2 (data not shown). It is interesting to note that citric acid and butyric acid were produced by *C. magnoliae*, which was opposed to production of ethanol in typical yeast fermentations with glucose. High concentrations of organic acids in the production stage did not inhibit metabolic activity for erythritol production since the specific erythritol production rate and erythritol conversion yield were well maintained at constant values throughout the whole period of the production stage (Figure 1).

Erythritol productivity (1.4 g l^{-1} h^{-1}) was obtained in the fed-batch culture process, which corresponded to a 2.6-fold increase compared with the previous batch culture [14]. This might be due to the combined effect of improved specific erythritol production rate from 0.025 to 0.042 g erythritol g $cells^{-1}$ h^{-1} and increased the average cell concentration from 26 to 85 g l^{-1} in the fed-batch culture. However, more than half of the glucose fed was converted into organic acids and cell mass in the production stage.

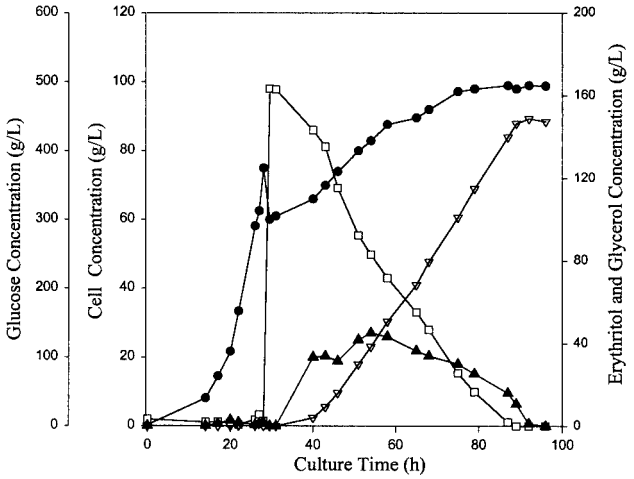

Figure 3 Profiles of fed-batch culture of *C. magnoliae* with 500 g l^{-1} of initial glucose concentration in the second stage. (□) Glucose; (●) cell mass; (▽) erythritol; (▲) glycerol.

Consequently, only 23% of erythritol conversion yield was obtained in the fed-batch mode.

Effects of initial glucose concentration in production stage

Variation of initial glucose concentrations in the production phase exerted a significant effect on erythritol production rate and erythritol conversion yield. For example, 400 g l^{-1} glucose in the production stage gave the results displayed in Figure 2. The first stage and cell growth profile in the production phase indicated a pattern similar to the results of 300 g l^{-1} glucose concentration (Figure 1). However, erythritol was produced at a rate of 4.9 g l^{-1} h^{-1} in the production stage and reached 187 g l^{-1} after 68 h of culture time. As a result, 2.8 g l^{-1} h^{-1} overall erythritol productivity and 41% of erythritol conversion yield were achieved, which corresponded to a 2.0-fold increase in erythritol productivity and a 1.8-fold increase in erythritol conversion yield compared with the case of 300 g l^{-1} glucose concentration. It was probably due to efficient utilization of glucose for erythritol production rather than for cell mass and organic acids (Figure 2).

The result of 500 g l^{-1} initial glucose concentration in the production stage is shown in Figure 3. An overall profile including butyric and citric acid formation was similar to that of 300 or 400 g l^{-1} glucose concentration. On the other hand, the level of glycerol formed sharply increased, to 43 g l^{-1}, and the erythritol production rate and erythritol conversion yield decreased to 3.1 g l^{-1} h^{-1} and 38% respectively, in the production phase. It appeared that the osmolarity of 2.5 OsM kg^{-1} due to a glucose concentration of 500 g l^{-1} in the culture broth reduced metabolic activity for erythritol production from glucose.

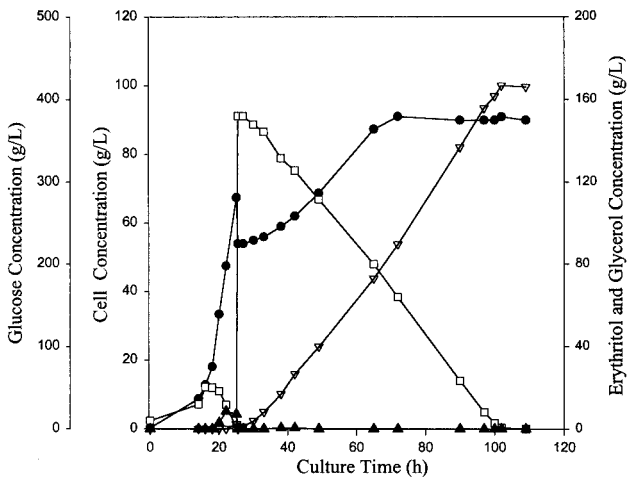

Figure 4 Two-stage fed-batch culture profiles with 40 g l^{-1} of yeast extract concentration in the first stage and 400 g l^{-1} of initial glucose concentration in the second stage. *C. magnoliae* was cultured at pH 7.0 and 28°C in a 3.3-l jar fermentor. (□) Glucose; (●) cell mass; (▽) erythritol; (▲) glycerol.

Effects of yeast extract

Yeast extract provides nitrogen and vitamin sources for cell growth and erythritol production. Effects of yeast extract concentrations were investigated in an attempt to improve the erythritol conversion yield. Yeast extract concentrations declined from 67 to 40 g l^{-1} in the growth stage (Figure 4). As the specific erythritol production rate was reduced from 0.051 to 0.032 g erythritol g $cells^{-1}$ h^{-1}, the erythritol production rate in the production stage also decreased from 4.9 to 2.3 g l^{-1} h^{-1}. The erythritol conversion yield was also slightly reduced, from 48% to 44% in the production phase. It was considered that efficient conversion of erythritol from glucose required a high level of key nutrients contained in yeast extract. An increase in yeast extract concentrations, from 67 to 80 g l^{-1}, in the growth stage did not change the erythritol conversion yield and erythritol productivity in the production phase (data not shown).

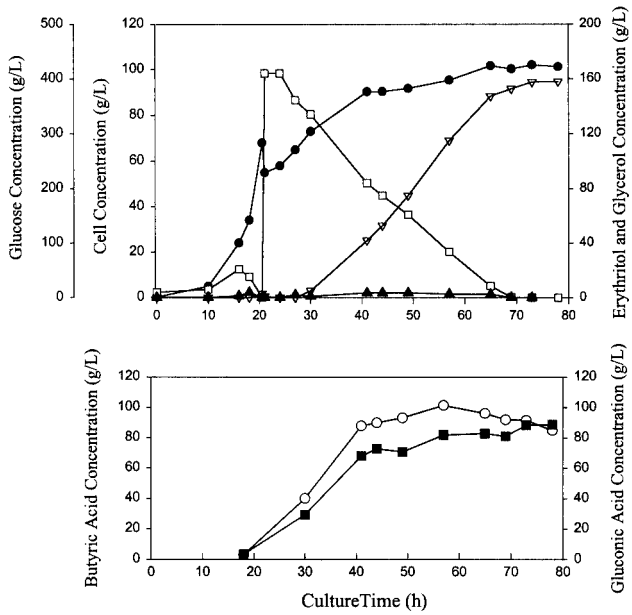

Figure 5 Experimental results of fed-batch culture of *C. magnoliae* with 400 g l^{-1} of initial glucose concentration and 40% dissolved oxygen in the second stage. (□) Glucose; (●) cell mass; (▽) erythritol; (▲) glycerol; (○) gluconic acid; (■) butyric acid.

Control of organic acid formation in production stage

With 400 g l^{-1} initial glucose concentration in the production stage, 137 g l^{-1} organic acids were produced. The erythritol conversion yield could be enhanced by controlling formation of organic acids in the production stage. The dissolved oxygen level in the culture broth increased from 20% to 40% saturation by using pure oxygen in the production phase. When a dissolved oxygen level was controlled at 20% saturation, 65 g l^{-1} citric acid and 54 g l^{-1} butyric acid were produced (Figure 2). The dissolved oxygen level increased to 40% and 96 g l^{-1} gluconic acid and 88 g l^{-1} butyric acid were formed in the production phase (Figure 5). It appeared that a portion of the carbon flux was shifted toward gluconic acid formation from citric acid production. Although butyric acid is known to be synthesized from pyruvate under anaerobic conditions, its production was not affected much by increasing the dissolved oxygen level. Erythritol conversion yield was the same regardless of the dissolved oxygen level.

More research on carbon flux analysis and its control is necessary in order to elucidate the carbon metabolism in *C. magnoliae* and to enhance erythritol conversion yield.

In conclusion, 2.8 g l^{-1} h^{-1} of overall erythritol productivity and 41% of erythritol conversion yield were achieved by optimization of cell growth and erythritol production conditions in a two-stage fed-batch process, which corresponded to a fivefold increase in erythritol productivity compared with the simple batch fermentation process.

Acknowledgements

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