JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 101, No. 1, 38–41. 2006
DOI: 10.1263/jbb.101.38

High-Cell-Density Fermentation for Ergosterol Production by *Saccharomyces cerevisiae*

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Received 10 August 2005/Accepted 19 October 2005

The direct feedback control of glucose using an on-line ethanol concentration monitor for ergosterol production by high-cell-density fermentation was investigated and the fermentation parameters (*e.g.*, pH, dissolved oxygen, ethanol concentration, oxygen uptake rate, carbon dioxide evolution rate and respiratory quotient) were analyzed. Controlling glucose feeding rate in accordance with ethanol concentration and adjusting pH with ammonia during the fermentation process were effective fed-batch methods for ergosterol production. The fermentation parameters well described the variation of the whole fermentation process. Cultivation in a 5 *l* fermentor was carried out under the following conditions: culture temperature, 30°C; pH, 5.5; agitation speed, 600 rpm; fermentation time, 60 h; controlling ethanol concentration below 1% and keeping respiratory quotient (RQ) at approximately 1.0. Under these conditions, the yeast dry weight reached 120 g/*l* and the ergosterol yield reached 1500 mg/*l*.

[**Key words:** ergosterol, *Saccharomyces cerevisiae*, biomass, high cell density, ethanol concentration, respiratory quotient, fermentation parameter]

Ergosterol, an important pharmaceutical intermediate, is the precursor of liposoluble vitamin D_2 and cortisone (1). It is also a main sterol in yeast cells and is responsible for structural membrane features such as integrity, fluidity, permeability and the activity of membrane-bound enzymes (2). Ergosterol is mainly produced by two different methods. First, ergosterol can be extracted from waste mycelium in penicillin or in citric acid fermentation. Second, ergosterol can also be produced by yeast fermentation. The raw materials are much cheaper in the former method, but the ergosterol content of mycelium is lower than that of yeast (3). Several common means of improving ergosterol yield include the screening of highly productive strains, the optimization of cultivation conditions and the overexpression of genes (*HMG1*, *ERG1*, *ERG9*, *ERG11*) in the ergosterol biosynthetic pathways of a mutant strain (4–6). Fed-batch fermentation is generally applied to achieve a more efficient production of proteins or other intracellular products by *Saccharomyces cerevisiae* (7). The limiting substrate concentration of carbohydrate must be maintained below the value that enables the maximum respiratory capacity of *S. cerevisiae* to overcome the repression caused by the oversupply of carbohydrate. The high concentration of ethanol during fermentation is unfavorable for the growth of yeast cells. On-line analysis of glucose (8) and oxygen uptake rate, and the measurement of respiratory quotient (RQ) (9) are used for feedback control of glucose limitation. Gao *et al*. (10) introduced a DO-control pulse fed-batch method, and the yield of ergosterol reached 1064 mg/*l*. However, it is feasible to employ a variable (ethanol concentration) that can be measured effectively for the feedback control of glucose feeding rate. Many types of on-line ethanol detector can be applied to analyze ethanol concentration in the fermentation liquor. Alfafara *et al*. (11) adopted a fuzzy control of the ethanol concentration fed-batch method for glutathione production and reached the maximum glutathione production. Indirect parameters (such as oxygen uptake rate, carbon dioxide evolution rate and respiratory quotient) during fermentation are also sensitive control variables, which require careful regulation. An efficient production of bakers' yeast has been traditionally performed by applying an indirect feedback control of RQ data to control glucose feeding rate. RQ should be controlled to a value of less than 1.3 (8). The application of RQ data as sensitive control variables for *S. cerevisiae* fermentation requires a careful design.

In our study, we monitored ethanol concentration for the feedback control of glucose feeding rate in ergosterol production by *S. cerevisiae* in a 5 *l* stirred-tank fermentor. Oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and RQ as secondary feedback control variables are calculated simultaneously. The relationship between direct fermentation parameters (pH, DO, biomass, ethanol concentration, glucose concentration and glucose feeding rate) and indirect fermentation parameters (OUR, CER and RQ) will be discussed.

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MATERIALS AND METHODS

Strain *S. cerevisiae* (Y-E-1), preserved in our laboratory, was used for ergosterol fermentation.

Medium The following media were used in this study. Plate medium (w/v) (%): yeast extract, 1; peptone, 2; industrial glucose, 2; and agar, 2; seed culture medium (w/v) (%): yeast extract, 1; peptone, 2; and industrial glucose, 2; fermentation medium: 60 g/*l* industrial glucose, 15 g/*l* corn steep liquor, 6 g/*l* KH_2PO_4, 3 g/*l* $MgSO_4$, 1 mg/*l* $CuSO_4 \cdot 5H_2O$, 1 mg/*l* $FeSO_4 \cdot 7H_2O$, and 10 mg/*l* $ZnSO_4 \cdot 7H_2O$; fed medium: 600 g/*l* industrial glucose solution.

Fed-batch fermentation Firstly, a plate culture of yeast cells was grown at 30±1°C for 20–24 h. Secondly, the yeast cells were transferred into a 250-ml flask containing 50 ml of seed culture medium, and the flask was incubated at 30±1°C and 180 rpm, for 20–24 h. The fermentor (Biotech-5BG; Shanghai Baoxin Bioengineering Equipment, Shanghai, China) containing 2 *l* of fermentation medium was sterilized at 121°C for 20 min and inoculated with 10% (v/v) seed cultures of yeast cells. Fermentation was carried out at 29.5±0.5°C, at an agitation speed of 600 rpm and air flow rate of 10 *l*/min. The dissolved oxygen was measured using an autoclavable O_2 sensor (Mettler Toledo, Greifensee, Switzerland). A solution of 600 g/*l* glucose was added into the reactor using the feedback control system and pH was adjusted to 5.5±0.1 by the automatic addition of 25% ammoniacal liquor.

Ergosterol extraction Five milliliters of fermentation medium (OD_{660} of 0.5, 1:50 dilution) was centrifuged (4000 rpm, 10 min) to separate the yeast cells. Then, the cells were transferred into a 100 ml flask followed by the addition of 8 g of KOH and 32 ml of 60% ethanol solution (v/v). For saponification, the mixture was treated for 2 h at 80°C in a water bath. Twenty-five milliliters of petroleum ether (boiling point range, 60–90°C) was added to extract sterol.

Analytical methods Dry cell weight was determined gravimetrically and showed a functional relationship to the spectrophotometrically measured turbidity at 660 nm. Ethanol concentration was determined on-line using an ethanol analysis instrument (East China University of Science and Technology, Shanghai, China). Glucose concentration was determined off-line using a biosensor (SBA-40C; Biology Institution of Shandong Academy of Science, Jinan, China). For on-line analysis, the oxygen and carbon dioxide contents of the exhaust gas produced from fermentation were analyzed using an exhaust gas analyzer (LKM2000-03; Lokas Automation Corp., Teajeon-City, South Korea). OUR, CER and RQ were calculated by following equations:

$$OUR = \frac{F_{in}}{V}\left[Co_{2\ in} - \frac{C_{in}^{\ inertia} * Co_{2in}}{1-(Co_{2out}+Cco_{2out})}\right] \quad (1)$$

$$CER = \frac{F_{in}}{V}\left[\frac{C_{in}^{\ inertia} * Cco_{2out}}{1-(Co_{2out}+Cco_{2out})} - Cco_{2in}\right] \quad (2)$$

$$RQ = \frac{CER}{OUR} \quad (3)$$

Where F_{in} indicates inlet gas flux (mol/h); V indicates fermentation liquid volume (*l*); $C_{in}^{\ inertia}$, Co_{2in}, and Cco_{2in} indicate the concentrations of inert gas, oxygen and carbon dioxide in the inlet gas, respectively, %; Co_{2out} and Cco_{2out} indicate the concentrations of oxygen and carbon dioxide in the outlet gas, respectively, %.

Ergosterol was quantified by HPLC (LC-10Atvp; Shimadzu, Kyoto; silica gel column, 4×250 mm; mobile phase: mixture of *n*-hexane and tetrahydrofuran [85:15, v/v]; flow rate, 1.0 ml/min with an ultraviolet photometric detector at 280 nm; ergosterol was purchased from Sigma-Aldrich China [Shanghai, China] [purity ≥98%] as standard). For simplicity, ergosterol was also quantified using a spectrophotometer (Unico Corp., Shanghai, China) at 280 nm.

Pyruvic acid in the fermentation broth was quantified by HPLC (LC-10Atvp; Shimadzu; Aminex HPX-87H ion exclusion column, 300×7.8 mm; mobile phase: 5 mmol/*l* H_2SO_4; flow rate, 0.6 ml/min; column temperature, 60°C; pyruvic acid was detected using an ultraviolet photometric detector at 210 nm).

RESULTS AND DISCUSSION

Trend of pH during fermentation The pH increased from 7th to 10th hour and decreased from 10th to 16th hour of fermentation (Fig. 1). With the depletion of the initial glucose during the early phase of fermentation (0–7 h), the yeast cells continued to grow by consuming ethanol and organic acid produced during the early phase of growth. The consumption of organic acid increased the pH. The feeding of glucose to the cells started immediately after the depletion of the initial glucose at the 10th hour. The yeast cells consumed glucose by aerobic metabolism and the accumulation of organic acid induced a gradual decrease in pH. Ammoniacal liquor (25%), which also served as nitrogen source, was used for pH control during the fed-batch fermentation. When the fermentation was finished, the yeast cells autolyzed and inclusions were released, causing an increase in pH.

Trend of DO during fermentation In the early phase of fermentation, DO decreased rapidly whereas OUR increased gradually with an increased consumption of oxygen (Fig. 2). Both air flow rate and agitation speed should be increased to maintain DO above 10%. Because of insufficient carbon sources, DO increased rapidly at the 10th hour and glucose was added in time. Increasing glucose feeding rate with the gradual reduction in ethanol concentration resulted in the high respiratory capacity of yeast cells. DO decreased again with the consumption of glucose and OUR increased from 0.25 to 5.5 mol/(h·*l*). DO varied inversely in proportion to OUR under well-ventilated conditions.

Trend of biomass during fermentation Owing to the high initial concentration of glucose, the yeast cells carried out their metabolism anaerobically with a relatively low energy yield. The yeast cells grown anaerobically had a relatively low growth rate coupled with a high rate of con-

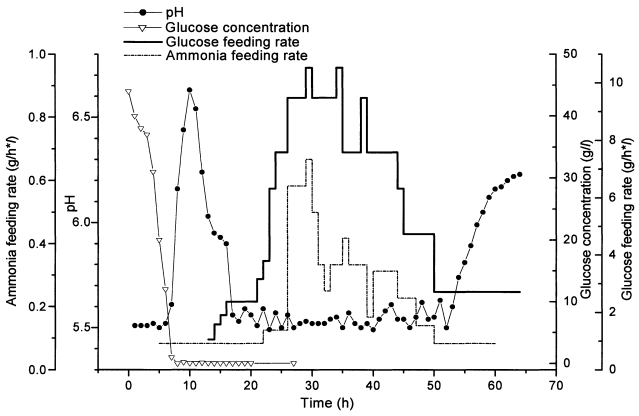

FIG. 1. pH trend line during fermentation. Solid circles, pH; inverted open triangles, glucose concentration; solid line, glucose feeding rate; short dotted line, ammoniacal liquor feeding rate.