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Exponential feeding strategy of high-density cultivation of a salt-tolerant aroma-producing yeast *Zygosaccharomyces rouxii* in stirred fermenter

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ABSTRACT

Salt-tolerant yeast *Zygosaccharomyces rouxii* is a key strain to produce flavor in soy sauce brewing. The aim of this research was to establish the optimal feeding strategy and parameter for high-density culture of *Z. rouxii*. An exponential feeding strategy was applied where the specific growth rate was fixed by limitation of the fed carbon source. The set point of specific growth rate of exponential feeding for fed-batch culture was chosen to be 0.03–0.07 h^{-1}. The optimal set point of specific growth rate (μ_{set}) of exponential feeding started after glucose depletion for fed-batch culture was found to be 0.05 h^{-1}. Furthermore, a relatively high-dense biomass (72.86 g/L dry cell weight) was obtained in the culture of exponential feeding started after glucose was exhausted with μ_{set} = 0.05 h^{-1}. Less than the level of *Saccharomyces cerevisiae* and *Pichia pastoris*, however the biomass concentration was much higher than data from other research about *Z. rouxii* culture.

1. Introduction

Soy sauce is one of the most popular Chinese traditional condiments that are produced in a fermentation process [1]. In the first step of the fermentation process, *Aspergillus sojae* or *Aspergillus oryzae* are grown on raw materials (steamed soybeans and roasted wheat) [2]. This culture is called koji. Then, brine fermentation, a mixture of koji and salt (NaCl) water, is fermented by salt-tolerant lactic acid bacteria and yeast [3]. In the soy sauce fermentation process, a salt-tolerant LAB, *Tetragenococcus halophilus* was found in soy sauce brewing mush, and salt-tolerant yeasts, such as *Zygosaccharomyces rouxii*, *Torulopsis versatilis* and *Candida versatilis* are very important for flavor formation [4]. Yeasts and LAB can grow using glucose produced by the diastatic enzymes of koji molds. *T. halophilus* can grow in highly salty soy sauce mash. After koji molds are mixed with brine in the mash, *T. halophilus* grows, lowering the pH of soy sauce mash by metabolizing the glucose to lactic acid. In turn, halotolerant yeast *Z. rouxii* becomes dominant, producing flavors, alcohol, and 4-hydroxy-2 (or 5)-ethyl-5 (or 2)-methyl-3(2*H*)-furanone (HEMF), a major fragrance associated with soy sauce [5].

The use of active dry yeast (ADY) in wine making is widespread. From a view of industrial point, ADY are practical, as the conservation of microbial cells in a dehydrated state makes it possible to keep their vitality for long times [6]. Using ADY in soy sauce fermentation is a novel strategy. High-cell-density cultivation techniques have been developed to achieve high final biomass and product yield in industrial cultivation [7]. Due to the Crabtree-positive in yeast fermentations, ethanol is formed when the substrate concentration is above a critical value [8]. This phenomenon is the so-called overflow metabolism [9]. Fed-batch culture can circumvent carbon overflow to ethanol by controlling the nutrient feeding. A number of nutrient feeding strategies in fed-batch culture of yeast cells have been reported, such as continuous feeding, pH stat, and dissolved oxygen (DO) stat. The pH- or DO-stat method is based on the observation that the pH or DO changes when the principal carbon substrate becomes depleted in culture media. When the pH or DO begins to rise due to the carbon source depletion, a predetermined amount of concentrated feeding solution is fed into the bioreactor [10]. Exponential feeding strategy (EFS) is an open-loop fed-batch

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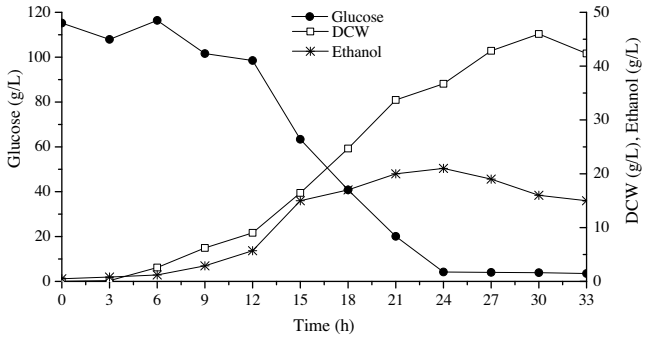

Fig. 1. Time course of cell growth in batch culture of *Z. rouxii* CCTCC M 2013310.

strategy which achieves a certain predetermined feeding profile. After a batch phase without feeding, an exponentially increasing feeding rate has to be applied in order to maintain a more or less constant growth rate [11].

This work described the application of controlling the specific growth rate through control of the substrate feed rate to achieve the high-cell-density cultivation of *Z. rouxii*. The cultivation a standard 10 L laboratory bioreactor was used and a group of specific growth rate was applied to calculate the substrate feeding rate.

2. Materials and methods

2.1. Yeast strain and culture media

Z. rouxii CCTCC M 2013310 was used in this study. The strain was maintained on YPDA medium (yeast extract, 10 g/L; peptone, 20 g/L; glucose, 20 g/L; and agar, 15 g/L). YPD medium which excluded agar in YPDA was used as seed culture medium. Fermentation medium established on preliminary work: glucose, 120 g/L; corn steep liquor, 23 mL/L; KH_2PO_4, 2.7 g/L; yeast extract, 10 g/L; glycerol, 30 mL/L; thiamine, 0.01 g/L. Fed medium: 600 g/L glucose and 56.7 g/L $(NH_4)_2SO_4$ mixed solution. Glucose and $(NH_4)_2SO_4$ were sterilized by autoclaving at 115 °C for 20 min, respectively.

2.2. Cultivation

The seed culture was prepared in a flask on a reciprocal shaker at 180 rpm and 28 °C for 30 h. Batch culture and fed-batch culture were carried out in a 10-L stirred tank reactor. 5.0% (v/v) seed culture was used to inoculate the fermentor. The initial volume of the culture was 6 L. Temperature was controlled at 28 °C. The aeration was maintained at 1.0 vvm and the agitation speed was operated at 500 rpm. The pH was keep at 5.0 by 4 mol/L NaOH. In fed-batch culture, feeding was started when glucose was exhausted and the feed rate was according to the exponential feeding profile.

2.3. Specific growth rate calculation

The specific growth rate achieved in batch phase was calculated using the DCW at each sampling point, according to the following equation [12]:

$$\mu = \left(\frac{1}{X}\right)\left(\frac{dX}{dt}\right) = \frac{d(\ln X)}{dt} \quad (1)$$

where X represents the mass of the cells at each sampling point (g/L).

2.4. Exponential feeding profile

In our study, exponential feeding strategy was applied after glucose was exhausted. The mass of glucose (the primary growth-limiting nutrient) added to the culture can be described using the equation [13,14]:

$$S_t = \frac{V_0 X_0 \left(e^{\mu_{set} t} - 1\right)}{Y_{x/s}} + S_0 \quad (2)$$

where S_t is the mass of glucose added to the culture at time t, S_0 the mass of glucose fed before time t, V_0 and X_0 the initial volume of medium (L) and concentration of biomass (g/L) at feeding time 0 h, respectively, $Y_{x/s}$ the biomass yield coefficient (g/g), μ_{set} the specific growth rate (h^{-1}). Based on batch fermentation analysis, the fed-batch culture commenced at X_0 = 33 g/L and V_0 = 6 L. By setting μ_{set} at a certain value, the feeding rate can be determined.

2.5. The yield and productivity

In order to better show the fermentation diversity between four feeding rate, the growth yield ($Y_{x/s}$), Ethanol yield ($Y_{eth/s}$), and Biomass productivity (g/(L h)) were particularly concerned during the feeding period. Three indicators is calculated by the following formula:

$$Y_{x/s} = \frac{\Delta(\text{cell dry weight})_{feeding}}{\Delta(\text{glucose})_{feeding}} \quad (3)$$

$$Y_{eth/s} = \frac{\Delta(\text{ethnaol})_{feeding}}{\Delta(\text{glucose})_{feeding}} \quad (4)$$

$$\text{Biomass productivity} = \frac{\Delta(\text{cell dry weight})_{feeding}}{\Delta(\text{time})_{feeding}} \quad (5)$$

where Δ(X) is the concentration or time difference between the feeding end point and the feeding starting point. If the difference is negative, the difference was zero.

2.6. Analytical methods

Samples were taken every 3 h manually. Optical density (OD) was measured by a spectrophotometer at 600 nm after proper dilution. Dry cell weight (DCW) was determined gravimetrically by centrifuging 5 mL samples at 8000 rpm for 10 min. Yeast cells were washed with distilled water and dried until constancy of weight was achieved. Ethanol and glucose concentration were determined off-line using a biosensor SBA-40C (biology Institution of Shandong Academy of Science, Jinan, China). Dissolved oxygen (DO) was monitored by the online DO electrode.

3. Result and discussion

3.1. Basic conditions of the feeding culture

Feeding medium is one of key parameters in batch fermentation research. Sole glucose as feeding fluid was not obvious raise the density of *Z. rouxii* CCTCC M 2013310 cell. The mixture of available carbon (600 g/L glucose) and nitrogen (56.70 g/L $(NH_4)_2SO_4$) sources was more suitable for feeding liquid of *Z. rouxii* in high-density culture process (data not show). Fed cultivation with a constant rate was an application mature feeding mode in a variety of microorganisms, but this strategy was not very appropriate for cultivation of *Z. rouxii* CCTCC M 2013310. The concentration of *Z. rouxii* CCTCC M 2013310 cell had slight increase from 45.98 to 52.42 g/L when the feeding liquid was added in fermenter at constant rate (1.0 mL/min) during the late exponential growth phase (data not show). *Z. rouxii* cell was not explosive growth during the constant speed feeding period because the requirement of cell nutrient and the supplementary of nutrient was inconsistent. To establish the optimum relationship between feeding rate and