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# Mixed culture of *Saccharomyces cerevisiae* and *Acetobacter pasteurianus* for acetic acid production

Zhi Wang[a], Mei Yan[a], Xiong Chen[a,*], Dongsheng Li[a], Li Qin[a], Zhijun Li[b], Juan Yao[b], Xinle Liang[c]

[a] Key Laboratory of Fermentation Engineering (Ministry of Education), Hubei Provincial Cooperative Innovation Center of Industrial Fermentation, College of Bioengineering, Hubei University of Technology, Wuhan 430068, PR China
[b] Hubei Province Key Laboratory of Yeast Function, Angel Yeast Co., Ltd., Yichang 433003, PR China
[c] College of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou 310018, PR China

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ABSTRACT

Mixed culture of *Saccharomyces cerevisiae* and *Acetobacter pasteurianus* was carried out for high yield of acetic acid. Acetic acid production process was divided into three stages. The first stage was the growth of *S. cerevisiae* and ethanol production, fermentation temperature and aeration rate were controlled at 32 °C and 0.2 vvm, respectively. The second stage was the co-culture of *S. cerevisiae* and *A. pasteurianus*, fermentation temperature and aeration rate were maintained at 34 °C and 0.4 vvm, respectively. The third stage was the growth of *A. pasteurianus* and production of acetic acid, fermentation temperature and aeration rate were controlled at 32 °C and 0.2 vvm, respectively. Inoculation volume of *A. pasteurianus* and *S. cerevisiae* was 16% and 0.06%, respectively. The average acetic acid concentration was 52.51 g/L under these optimum conditions. To enhance acetic acid production, a glucose feeding strategy was subsequently employed. When initial glucose concentration was 90 g/L and 120 g/L glucose was fed twice during fermentation, acetic acid concentration reached 66.0 g/L.

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## 1. Introduction

Traditional fermentation process to produce vinegar includes three independent processes, which consists of saccharification of the rice or other starch materials by available enzymes from mold, ethanol fermentation by yeast and acetic acid fermentation by acetobacter. Practically, three different microorganisms, such as molds, yeasts and acetobacters are used in these independent biochemical stages in order to complete starch saccharification, ethanol fermentation and acetic acid fermentation. These three biochemical processes have different optimal conditions, which result in a long and complex process management in the traditional vinegar production [1–4].

Mixed culture systems exhibit great advantages for many processes involving more than one reaction step, such as high productivity, simple technique and low capital investment [5–9]. With a mixed culture system, multiple reaction steps can be finished in a single fermentor [10]. Mixed culture fermentation may become an attractive addition to traditional pure-culture-based biotechnology for the traditional fermented food [11]. However, it is difficult to use mixed culture fermentation in industrial scale, because various microorganisms present in a mixed culture system usually have different optimal culture conditions such as pH, temperature, substrate, and oxygen concentrations. A favorable culture condition for one microorganism may even be inhibitory for another microorganism within the system of mixed culture. It is therefore difficult to determine the culture condition that can equally support maximum activities of the various microorganisms [10].

Although the advantages of mixed culture over pure culture have been pointed out by many researchers [12], little is known about the application of mixed culture technology in acetic acid production [13]. Recently, we have applied mixed culture to produce acetic acid in a shake flask. Acetic acid concentration was 89.3 g/L. In this study, the possibility to produce acetic acid with mixed culture fermentation was evaluated in a stirred tank reactor (STR); the process parameters were optimized by an orthogonal experimental design; and fed-batch culture was applied to enhance the yield of acetic acid production.

## 2. Materials and methods

### 2.1. Microorganisms

Acetobacter is *Acetobacter pasteurianus* CICC7015 from China Center of Industrial Culture Collection. Yeast is super alcohol active

* Corresponding author. Tel.: +86 27 88032320; fax: +86 27 88032320.
*E-mail address:* cx163_qx@163.com (X. Chen).

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dry yeast (*Saccharomyces cerevisiae*) from Angel Yeast Company Ltd. (China).

### 2.2. Culture medium

Seed medium for *A. pasteurianus* contained: ethanol 4% (v/v), yeast extract 1%, $MgSO_4{\cdot}7H_2O$ 0.02%, $K_2HPO_4$ 0.1%. Fermentation medium contained: glucose 9%, yeast extract 1%, peptone 0.2%, $KH_2PO_4$ 0.1%, $K_2HPO_4$ 0.1%, $MgSO_4{\cdot}7H_2O$ 0.02%, sodium glutamate 0.4%.

### 2.3. Culture method

For seed culture of acetobacter, a loopful of slant culture of *A. pasteurianus* was inoculated into a 250 mL flask containing 50 mL seed medium, precultured statically at 30 °C for 48 h and then 50 mL seed culture was inoculated into a 1,000 mL flask containing 500 mL seed medium and statically cultured again at 30 °C for 48 h. It was used as a seed culture for batch and fed-batch cultures. For seed culture of yeast, 3% glucose and 2% active dry *S. cerevisiae* were mixed and stirred for 30–50 min at 30 °C until bubble appeared. It was also used as a seed culture for batch and fed-batch cultures.

For batch fermentation, a 10 L fermentor (type SGJ-10L/C; Bao Xing Co. Ltd., Shanghai) was used. Based on experimental data before, agitation rate was 100 rpm and dissolved oxygen concentration (DO) was kept at above 20%. Acetic acid process was divided into three stages according to the characters of growth and metabolization of *S. cerevisiae* and *A. pasteurianus*: the first stage for growth of *S. cerevisiae*, the second stage for co-culture of *S. cerevisiae* and *A. pasteurianus*, the third stage for growth of *A. pasteurianus*. Fermentation temperature and aeration rate were controlled differently at these stages. Inoculation amount of acetobacter and yeast, fermentation temperature and aeration rate were optimized by an orthogonal experimental design.

For fed-batch culture, glucose was fed when the glucose concentration in the culture broth was lower than 5 g/L, and the total glucose addition was 210 g/L.

### 2.4. Orthogonal array design

According to the result from single factor optimization in the earlier study, inoculation amount of *A. pasteurianus* and *S. cerevisiae*, fermentation temperature and aeration rate were used as the factors to further optimize. A $L_9(3^4)$ orthogonal experimental design with four factors, each factor with three different levels, was used in the study (Table 1).

### 2.5. Acetic acid and ethanol assay

Acetic acid and ethanol was determined by gas chromatography. An AT-Wax capillary column was used in GC system. The parameters were set as follows: carrier gas nitrogen, flow rate of carrier gas 10 mL/min, split ratio 10:1, temperature of injection port 220 °C, temperature of detector 260 °C. The column temperature program, firstly kept at 50 °C for 3 min, secondly heated up to 80 °C by the rate of 10 °C/min, then immediately heated up to 200 °C by the rate of 30 °C/min and then kept at 200 °C for 1 min.

Sample pretreatment was carried out as follows: 5 mL of samples were centrifuged at 10,000 rpm for 3–5 min. The supernatant was diluted to suitable concentration by decuple dilution and filtrated through 0.22 μm microporous membrane. Then 1.0 mL of each sample was mixed with 10 μL of isobutanol as internal standard. Standard sample was the mixture of 0.02% isobutanol, 0.02% ethanol and 0.02% acetic acid. When GC worked stably, samples were injected with the volume of 1 μL.

### 2.6. Glucose assay

Glucose biosensor instrument (SBA-40C, Shandong Academy of Sciences) was applied to detect glucose content in fermentation broth. Samples were diluted to an appropriate concentration to ensure an effective measurement range of the concentration. When instrument worked stably, samples were injected with the volume of 1 μL.

### 2.7. Plate number of acetobacter and yeast

Samples were aseptically diluted to suitable concentration by decuple dilution. Volumes of 0.1 mL of diluted samples in different concentrations were spread on the acetobacter medium including bromocresol green for *A. pasteurianus* and PDA medium for *S. cerevisiae*, respectively. The number of CFU/mL (1 CFU = 1 Colony-Forming Unit) was counted according to the dilution factor and the number of colonies on the plates with 30–300 colonies after incubation for 5 d at 30 °C for *A. pasteurianus* and for 48 h at 30 °C for *S. cerevisiae*, respectively.

## 3. Results and discussion

### 3.1. The preliminary experiment with mixed culture fermentation in stirred tank reactors

Recently, we have applied mixed culture to produce acetic acid in a shake flask. Through single factor experiment, the optimal process parameters were 6% inoculation amount of *A. pasteurianus*, 0.15% inoculation amount of *S. cerevisiae*, 15% glucose concentration, 30 °C fermentation temperature. Acetic acid concentration was 89.3 g/L under these optimal conditions. Based on these experimental data, mixed culture of yeast–acetobacter.in a fermentor with a maximum working volume of 10 L was carried out with 0.05 Mpa cans pressure at 0.1 vvm aeration rate. These culture parameters were not changed till acetic acid concentration did not further increase. Samples from broth were taken daily to analyze glucose concentration, biomass, ethanol content and acetic acid content. The time courses of acetic acid production, the cell growth and the changes of glucose and ethanol content were shown in Fig. 1. Acetic acid production was increased from 2 g/L to 16 g/L in the whole fermentation process, which was lower than that in vinegar. Glucose, as a carbon source, was rapidly utilized within 20 h. At the same time, ethanol content was increased remarkably

**Table 1**
Factors and their levels of orthogonal experiment $L_9$ ($3^4$).

| Constituent | Symbol | Level 1 | Level 2 | Level 3 |
|---|---|---|---|---|
| Fermentation temperature (°C) | *A* | 30:32:30[a] | 30:34:30 | 32:34:32 |
| Aeration rate (vvm) | *B* | 0.1:0.3:0.1[a] | 0.2:0.3:0.2 | 0.2:0.4:0.2 |
| Inoculation volume of *A. pasteurianus* (%) | *C* | 12 | 14 | 16 |
| Inoculation volume of *S. cerevisiae* (%) | *D* | 0.06 | 0.08 | 0.10 |

[a] The acetic acid fermentation was divided into three periods: the first stage for the growth of *S. cerevisia* and ethanol production between 0 and 24 h, the second stage for the co-culture of *S. cerevisiae* and *A. pasteurianus* between 24 h and 72 h, the third stage for the growth of *A. pasteurianus* and acetic acid production after 72 h. Different temperature and aeration rate would be controlled at different periods.

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