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Achieving high strength vinegar fermentation via regulating cellular growth status and aeration strategy

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ABSTRACT

Implementing of high strength vinegar fermentation is still the mission of vinegar producers. The aim of this study was to carry out high acidity vinegar fermentation efficiently based on comprehensive analysis on bacterial fermentation kinetics characteristics of *Acetobacter pasteurianus* CICIM B7003-02. In practice, semi-continuous vinegar fermentation was optimized with an optimal discharge/charge ratio of 34% of working volume (v/v), which resulted in a proper growth status of *Acetobacter* and beneficial to acetification. Then, a two-stage aeration protocol was adopted in the vinegar fermentation in line with the *Acetobacter* theoretical oxygen demand, by which both vinegar stoichiometric yield and acetification rate were improved effectively. As the final result, a titer of 93.09 ± 0.24 g/L acetic acid was achieved, the average acetification rate was enhanced to a level of 1.83 ± 0.01 g/L/h, and the vinegar stoichiometric yield was promoted to 93.97 ± 0.16%. The strategy and practice worked out from this study provided a valuable reference for performing large scale vinegar fermentation with higher strength.

1. Introduction

Vinegar has been widely used in food industry [1]. Global vinegar demand is increasing significantly in the recent ten years (http://www.versatilevinegar.org). Producing high acidity vinegar (acidity ≥ 90 g/L acetic acid) is one of the solutions to meet market demand, and it can also save storage and transport cost. *Acetobacter* as the main genus is involved in conventional rice wine vinegar production where acidity commonly does not exceed 60 g/L [2–4]. At present, few *Aectobacter* species were applied in high acidity rice wine vinegar production. So, how to ferment high acidity vinegar by *Acetobacter* species is valuable.

Vinegar is formed by the stoichiometric conversion of ethanol with oxygen to acetic acid and water by acetic acid bacteria (AAB) [5]. The advanced technology for this industry is based on the submerged fermentation in semi-continuous mode [6,7]. It consists of the developing of successive repeated acetification processes: when the concentration of alcohol reaches minimum residual level (depending on the type of vinegar), a quantity of medium is discharged and refilled with the same volume of fresh medium containing a certain amount of alcohol; the residual culture broth serves as inoculum and a new fermentation batch starts [8]. Seeking for high strength vinegar fermentation is always the mission of vinegar producers. Generally speaking, high strength vinegar fermentation means acetification process with acetification rate and vinegar stoichiometric yield at high level. Many technical devices and modifications have been tried to optimize vinegar fermentation [9–13]. Among these advances the Frings acetator technology is the most common application for the commercial production of all kinds of vinegar. Energy consumption is just 400 W/L ethanol which is much lower than aerated-stirred fermentors, and the maximum vinegar stoichiometric yield is as high as 95%. Commercial sizes of acetators are sufficient for the conversion of up to 3600 L pure ethanol in 24 h. The Frings alkograph installed in the acetator is an automatic instrument for online measuring the amount of ethanol in the fermenting liquid, which guarantees the smooth running of high strength vinegar fermentation [14].

In vinegar fermentation, acetification is directly associated with bacterial alcohol oxidation ability which is mainly determined by cellular growth status of AAB. In semi-continuous method, cellular growth status could be regulated by discharge/charge operation [15]. The most common operation for high acidity vinegar production by a few *Gluconoacetobacter* species is removing 40–50% (v/v) of the working volume [1,14]. However, such volume may not be suitable for species from the genus *Acetobacter*. Therefore, the optimal volume for *Acetobacter* should be obtained from practical

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fermentation trials. Oxygen supply measured by oxygen transfer rate (OTR) also influences cellular growth status. To guarantee fermentation performing smoothly, oxygen supply should meet bacterial oxygen consumption at least. Increasing aeration rate in a proper range will promote the OTR obviously. Nevertheless, a fast aeration rate will also speed up the evaporation of alcohol and acetic acid which results in vinegar stoichiometric yield reduction [16]. In general, losses of ethanol due to evaporation in industrial fermentation result in overall reductions of 10–30% compared to the stechiometric yield [17]. Thus, finding a balance between aeration rate and vinegar stoichiometric yield is necessary.

In the presented work, semi-continuous fermentations were carried out with various discharge/charge volumes after a proper starting-up process which guaranteed subsequent repeated-batches running smoothly [18]. In the fermentation experiments, trends of substrate/product concentration, cells growth, and dissolved oxygen were detected. Then, fermentation kinetic analysis was performed on these data. On the basis of the secondary processing data, an optimal discharge/charge operation could be achieved, by which both vinegar stoichiometric yield and acetification rate might be at a high level. In addition, a new index, ratio of oxygen consumption versus acid yield, was introduced to evaluate oxygen utilized efficiency [19]. According to the index aeration strategy was readjusted to improve vinegar stoichiometric yield further. As a result, an optimal operation could be raised for high acidity vinegar fermentation in semi-continuous mode.

2. Materials and methods

2.1. Strain, medium and culture condition

Acetobacter pasteurianus CICIM B7003-02, an ultraviolet mutant strain from *A. pasteurianus* CICIM B7003 (former name: *A. pasteurianus* FS1) [19], was used in the study. It can tolerate 60 g/L initial acidity and finally accumulate approximately 100 g/L acetic acid in shaking flask when fermentation proceeded for 120 h. In the study three kinds of media were used: YG_1 as seeds medium (L) (10 g yeast extract, 10 g glucose were dissolved in deionized water, natural pH); YG_2 as fermentation medium (L) (5 g yeast extract, 5 g glucose, 0.4 g $MgSO_4$ and 0.6 g KH_2PO_4 were dissolved in deionized water, natural pH); and YG_3 (L) as the solid medium that 20 g agar and 15 g $CaCO_3$ were added to YG_1. Anhydrous ethanol and anhydrous acetic acid were added aseptically into medium before inoculating. Chemicals used in this experiment were provided by Songong Biotech (Shanghai) Co., Ltd.

A. pasteurianus CICIM B7003-02 was inoculated into unbaffled Erlenmeyer flasks (250 mL) with YG_1 medium (50 mL) containing 32 g/L alcohol and 10 g/L acetic acid. These inoculated media were cultured in a THZ-22(8) bench constant temperature oscillator (Qiangle Laboratory Equipment Co., Ltd., Taicang, China) at 170 rpm and 30 °C for 24 h as seeds, approximately 6×10^8 colony forming units per mL (CFU/mL).

2.2. Starting-up process

Both the starting-up process and fermentation were carried out in a Frings 9 L Pilot-Acetator whose maximum working volume was 8 L (Heinrich Frings GmbH & Co KG, Bonn, Germany). The bioreactor is operated in automated manners (discharge, charge and monitored without operator intervention via previous programmed computer software).

A starting-up process should be initiated before semi-continuous fermentation. Fig. 1 showed the detailed schematic diagram of starting-up process. The process included four steps. Step-1, 1.8 L YG_2 medium containing 36 g/L alcohol and 12 g/L acetic acid were poured into the bioreactor and mixed adequately with 0.2 L seeds. Aeration rate was set at 21 L/h (0.175 vvm) for the first step. The step went on for about 19 h; step-2, 2 L YG_2 medium with 74 g/L alcohol were supplemented into the fermentor within 1 h to continue the starting-up process. Simultaneously, air flow rate was increased to 43 L/h (0.18 vvm). The second step continued for 17 h; step-3, after 2 L YG_2 medium with 90 g/L alcohol being replenished, the third step was started. Aeration rate was changed to 72 L/h (0.2 vvm). After performing for 20 h, the step was accomplished with approximately 69 g/L acetic acid accumulation; step-4, 2 L YG_2 medium with 90 g/L alcohol were fed to start the fourth step, and air flow rate was adjusted to 96 L/h (0.2 vvm). This step lasted for 22 h. Temperature was kept at 30 °C for the whole starting-up process. After the fourth step an intact starting-up process was completed. The acetator was finally filled with 8 L medium with a huge biomass population in the late exponential phase. During the process, cells growth was in the late exponential phase when fresh medium was added at each step. Such operation is helpful to obtain high quality biomass that cells growth

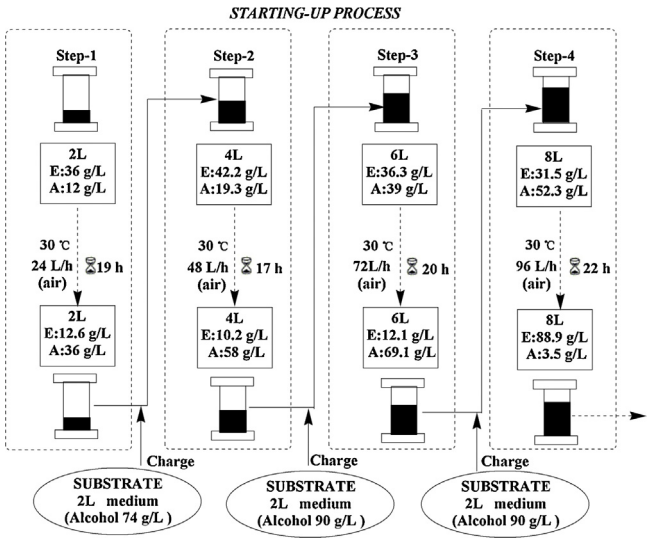

Fig. 1. Starting-up protocol for semi-continuous fermentations in a Frings 9 L Pilot-Acetator.

is up to the point of achieving an important cell population which is necessary to start the acetification process.

2.3. Semi-continuous fermentation

Semi-continuous fermentation was initiated when the starting-up process was completed. For the fermentation, a fraction of broth was withdrawn on a periodic basis and the remaining was replenished with the same volume of fresh medium to start a new batch. The detail was that after discharging a certain quantity of the total volume (40%, 34% and 29%, v/v) a new batch cycle was implemented by adding the same volume of fresh YG_2 medium with 76 g/L alcohol. Then, an acetification process was occurred as the previous one. Each repeated-batch was ended when the alcohol concentration was below 4 g/L. After four batch cycles running, a complete maintenance procedure was applied to the bioreactor (cleaning of vessel and sensors, repairing of pumps, etc.). Temperature was set at 30 °C and aeration rate was kept 96 L/h for all experimental groups.

2.4. Fermentation assay

Bacterial metabolism was detected by measuring change of alcohol, acidity and biomass during the fermentation. The acidity of the medium was measured by 0.1 M NaOH with phenolphthalein as pH indicator. Ethanol was recorded by ALKOSENS on line producted by Frings Company. Oxygen concentration was determined with a Clark oxygen electrode using a 53YSI oxygen-meter [20,21]. Biomass was revealed by colony forming units with plate cultivation. The detailed method: cells taken from various sampling points were immediately serially diluted in sterile saline (0.85%, w/v) and 0.1 mL of diluted samples was plated on YG_3 and colony forming units were determined after aerobic incubation at 30 °C until the distinct colonies appeared. All these experiments were performed in triplicate. Vinegar stoichiometric yield means the conversion rate of acetic acid from alcohol and is calculated as the following equation:

$$\text{Stoichometric yield}\,(\%) = \frac{Y_{acid}}{C_{alcohol} \times 1.304} \times 100\%$$

where Y_{acid} is the concentration of acetic acid in a medium (g/L), $C_{alcohol}$ is the concentration of alcohol disappeared in the same medium (g/L).

In this study, all experiments were repeated in three times. Statistical significance ($P \leq 0.05$) was determined using the SAS statistical analysis program, version 8.01 (SAS Institute, Cary, NC, USA). The data shown in the corresponding tables and figures were the mean values with the standard deviation.

3. Results and discussion

3.1. Characters of cells growth in different semi-continuous fermentations

Acetification is coupled well with cells growth during vinegar fermentation. Thus, cellular growth status was analyzed primarily for performing measurement of acetification process. Growth curves of the three experiment groups were shown in Fig. 2.

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