Journal of Bioscience and Bioengineering
VOL. 109 No. 1, 41 – 46, 2010

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Continuous ethanol fermentation from non-sulfuric acid-washed molasses using traditional stirred tank reactors and the flocculating yeast strain KF-7

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Received 29 April 2009; accepted 8 July 2009
Available online 28 July 2009

Waste molasses is one of the most important feedstock for ethanol production in Brazil as well as in many Southeast Asian countries, including China. Sulfuric acid pretreatment is employed in most ethanol distilleries in China to control bacterial contamination, which results in difficulties in the treatment of wastewater containing high levels of sulfate ions. In this study, a high efficiency, non-sterilized, continuous ethanol fermentation process without sulfuric acid pretreatment was developed using the flocculating yeast strain KF-7 and the widely utilized, traditional, stirred tank reactors. An alternative molasses medium feeding method, which differs from traditional methods, is proposed that effectively controls bacterial contamination. Separate feeding of 1.2-fold diluted molasses and tap water into the reactor proved to be effective against bacterial contamination during long-term continuous fermentation. By feeding yeast cells with high metabolic activity to the second reactor, a two-stage continuous fermentation process that yielded a high ethanol concentration of 80 g/l as well as high ethanol productivity of 6.6 g/l/h was successfully operated for more than one month. This fermentation process can be applied to ethanol distilleries in which traditional tank reactors are used.

[**Key words:** Molasses; Stirred tank reactor; Continuous ethanol fermentation; Flocculating yeast; Bioethanol production]

Sugar cane molasses is an abundant agro-industrial byproduct in Brazil and other tropical countries, including the southern part of China. Molasses is an important and economical substrate for ethanol distilleries due to its easily fermentable sugars and low cost.

We have developed non-sterile continuous fermentation processes using tower-type reactors (1, 2). Since flocculating yeast was used in these processes, and the yeast cells effectively accumulated in the reactor, it was possible to operate the system at a high dilution rate, resulting in a high ethanol concentration and high productivity. Most importantly, due to the high dilution rate and the large quantity of cells with high activity, bacterial contamination was successfully repressed and long-term non-sterile continuous fermentation was easily realized. However, the general application of these processes is difficult, since it is not feasible for traditional ethanol distilleries to construct new tower-type reactors.

Almost all traditional ethanol distilleries in China use tank reactors. Since the process currently employed by many distilleries is not very efficient, the continuous fermentation system is always operated at a relatively low dilution rate of approximately 0.02 h^{-1} and, in addition, cell numbers in the reactors are low, generally around $1–2\times10^8$ cells/ml. Furthermore, unless anti-contamination measures are taken, contamination is very likely to occur. In China, most distilleries employ sulfuric acid pretreatment as a sterilization method, resulting in an ethanol concentration of over 80 g/l and an ethanol productivity of about 1.5–2.0 g/l/h. Although this treatment is effective for contamination control, it results in the accumulation of about 5000 mg/l of sulfate ions in distillation wastewater, making it difficult to treat the wastewater (3–5). Although, previously, some large distilleries effectively treated wastewater by high-cost evaporation followed by combustion, most of the smaller distilleries could not, and, ultimately, most of these small distilleries were closed down. A continuous ethanol fermentation process without sulfuric acid pretreatment is of great importance for these types of distilleries.

In this study, which used the flocculating yeast strain KF-7 (6), a highly efficient, continuous ethanol fermentation process without sulfuric acid pretreatment was developed for ethanol distilleries in which traditional stirred tank reactors are utilized. A new molasses medium feeding method was proposed to control bacterial contamination and was verified to be effective. Several continuous fermentation processes, including or excluding feeding cells with high activity, were then investigated with the aim of achieving a high ethanol concentration as well as high ethanol productivity.

MATERIALS AND METHODS

Yeast strain used The flocculating yeast *Saccharomyces cerevisiae* KF-7 was used in this study. This yeast was constructed by protoplast fusion of the flocculating yeast strain IR-2 (7) and the thermotolerant yeast strain EP-1 (6).

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doi:10.1016/j.jbiosc.2009.07.002

Medium for yeast growth YPD medium containing 5% glucose, 1% yeast extract and 2% peptone was used for seed cultivation. The molasses used in this study was from Indonesia and the average total sugar concentration was 0.54 g/g. As a nitrogen source, 0.01 g of $(NH_4)_2SO_4$ was added per g molasses. Tap water was used to dilute the molasses. The only media that was sterilized was the YPD.

Preparation of the inoculum Stored yeast cells were inoculated into 100 ml of 5% YPD medium in a 500 ml flask. Pre-cultivation was performed aerobically at 30 °C for 16 h with mixing using a rotary shaker and a speed of 160 rpm. The resulting pre-cultivation broth was used as the inoculum for continuous fermentation.

Determination of the molasses medium feeding method The molasses was diluted 1.2-, 1.5-, 2- or 3-fold with tap water. Each dilution of the molasses was assayed as to its feeding potential by feeding via a peristaltic pump. Concurrently, each dilution of molasses was incubated at room temperature to investigate the extent of contamination. The concentration of volatile fatty acids (VFA) was measured at 0 h, 12 h and 48 h. Ethanol fermentation of each dilution of molasses was simultaneously performed by batch fermentation using 300 ml Erlenmeyer flasks. The concentration of ethanol was determined every 12 h.

Continuous ethanol fermentation Five continuous fermentation processes were studied and compared to determine which process for ethanol production yielded the highest ethanol concentration and had the highest ethanol productivity. All the magnetic stirred tank reactors used in the study were manufactured by Marubishi (Tokyo, Japan). The temperature, the aeration rate and the stirring rate for each reactor were controlled at 30 °C, 0.15 vvm and 150 rpm, respectively, unless otherwise specified. The concentration of ethanol, residual sugar and volatile fatty acids, as well as the total cell number in each reactor, was measured every day during the operation.

Process 1 was a one-stage fermentation process consisting of one stirred tank reactor (working volume 2.0 l) and one settler for yeast cell sedimentation, as shown in Fig. 1. Tap water and 1.2-fold diluted molasses were separately fed by peristaltic pumps to the reactor (hereafter named R1) at rates of 133 ml/h and 67 ml/h, respectively, so that the sugar concentration in the influent was 150 g/l. The dilution rate of R1 was 0.1 h^{-1}. The fermented broth was pumped to the settler and the yeast cells were returned to the reactor at a rate of 50 ml/h. Fermented broth was overflowed from the settler.

Process 2 was a two-stage fermentation process consisting of two stirred tank reactors and one settler, as shown in Fig. 2. Tap water and the 1.2-fold dilution of molasses were separately fed by peristaltic pumps to the first reactor (hereafter named R1, working volume 2.0 l) at rates of 133 ml/h and 67 ml/h, respectively, so that the sugar concentration in the influent was 150 g/l. The fermented broth from R1 was pumped to the second reactor (R2, working volume 3.0 l), and 1.2-fold dilutions of molasses were simultaneously fed to R2 at a rate of 22 ml/h, so that the total sugar fed to R2 reached 180 g/l, assuming that no sugar was consumed in R1. The fermented broth from R2 was pumped to the settler and the yeast cells were recycled to R1 at a rate of 50 ml/h. Fermented broth was overflowed from the settler. The dilution rates of R1 and R2 were 0.1 h^{-1} and 0.074 h^{-1}, respectively. The dilution rate of the total process was calculated to be 0.044 h^{-1}.

Process 3 was a two-stage fermentation process consisting of two stirred tank reactors and two settlers. The process was similar to Process 2, except that a settler was attached after both R1 and R2, and the yeast cells from the fermented broth of R1 and R2 that had settled were separately recycled back to R1 and R2, respectively, at rates of 50 ml/h. Other operation parameters were the same as in Process 2.

Process 4 was a two-stage fermentation process comprised of three stirred tank reactors and two settlers, as shown in Fig. 3. R1 was operated under the same conditions as for Processes 1 and 3. The fermented broth that overflowed from settler-1 was fed to R2. At the same time, the fermented broth from R3, a reactor for the cultivation of yeast cells with high activity, was fed to R2 at a rate of 200 ml/h. A 1.2-fold dilution of molasses was supplied to R2 at a rate of 98.3 ml/h so that the total sugar fed to R2 reached 180 g/l, assuming that no sugar was consumed in R1 and R3. The fermented broth from R2 was pumped to settler-2, and the settled yeast cells were returned to R2 at a rate of 50 ml/h. Fermented broth was overflowed from settler-2. The working volumes of R1, R2 and R3 were 2.0 l, 3.0 l and 1.0 l, respectively, and the dilution rates of R1, R2 and R3 were 0.1 h^{-1}, 0.166 h^{-1} and 0.2 h^{-1}, respectively. The dilution rate of the total process was calculated to be 0.083 h^{-1}.

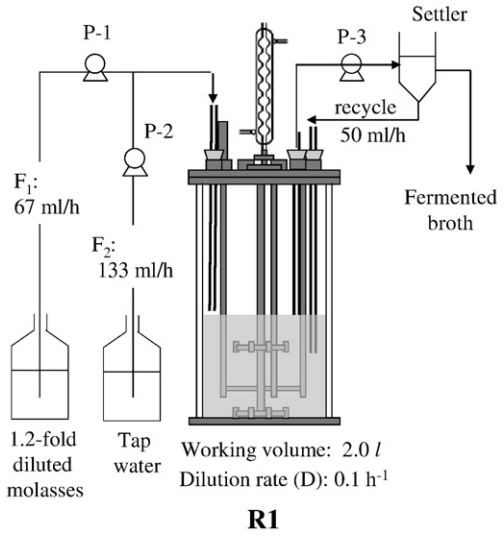

FIG. 1. Schematic diagram of the continuous fermentation process, Process 1.

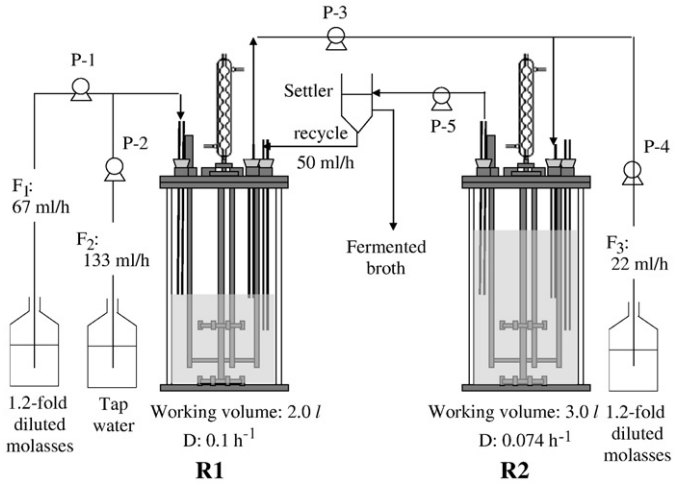

FIG. 2. Schematic diagram of the continuous fermentation process, Process 2.

Process 5 was a one-stage fermentation process consisting of two stirred tank reactors and one settler, which could be considered the same as Process 4 without R1. R3 served as a cell cultivation reactor, and R2 was the ethanol fermentation reactor. By feeding R2 with a 1.2-fold dilution of molasses at a rate of 60 ml/h, the total sugar fed to R2 was set at 180 g/l, assuming that no sugar was consumed in R3. The dilution rates of R2 and R3 were 0.087 h^{-1} and 0.2 h^{-1}, respectively. The dilution rate of the total process was calculated to be 0.065 h^{-1}.

Effect of sugar concentration and aeration rate on cell activity The optimum conditions for the cultivation of cells with high activity were determined for the operation of R3 in Processes 4 and 5. A stirred reactor with a working volume of 1.0 l was used for the continuous cultivation of the cells under different conditions. The dilution rate of the reactor was controlled at 0.2 h^{-1}. The total concentrations of sugar in the influent were set at 80 g/l, 100 g/l, 120 g/l and 150 g/l by feeding different ratios of tap water and 1.2-fold dilutions of molasses. Cell activity, as well as cell number and cell survival rate, was measured at each sugar concentration when the system reached a steady state. The effect of aeration on cell activity was investigated at a sugar concentration of 90 g/l. The aeration rate was varied from 0 to 0.15 vvm, and cell activity and the concentration of intracellular trehalose were measured at each aeration rate.

Analytical methods The total sugar concentration was assayed by the Somogyi–Nelson method after hydrolysis of the samples with 1% HCl (8). Ethanol concentration was measured by gas chromatography using isopropanol as the internal standard (9). Volatile fatty acids (VFAs) including lactic acid and acetic acid were analyzed by high performance liquid chromatography (HPLC) (10). The concentration of intracellular trehalose in yeast cells was measured as previously described (11).

The metabolic activity of yeast cells was assayed by measurement of the CO_2 production rate using Warburg's manometric method. The measurement was carried out at 30 °C with 1 ml of 1 M glucose solution and 5×10^7 cells suspended in 1 ml of 0.01 M KH_2PO_4 solution. The volumetric oxygen transfer coefficient (k_La) was determined by the oxygen balance method. The quantity k_La was calculated from the following equation: k_La (h^{-1}) = oxygen consumption rate (g/l/h) / ($C_0^* - C$), where, C_0^* is the saturated dissolved oxygen concentration in the broth (g/l) and C is the dissolved oxygen concentration in the broth (g/l). The oxygen consumption rate was calculated from the following equation: oxygen consumption rate (g/l/h) = air feeding rate (l/l/h) × (oxygen ratio in the influent air − oxygen ratio in the effluent air) × 32/22.4.

The total number of yeast cells and viable cells was calculated by the methylene blue method using a hematitometer.

RESULTS AND DISCUSSION

Determination of the molasses feeding method for continuous ethanol fermentation The traditional method of feeding molasses medium for continuous ethanol fermentation in most of the ethanol distilleries in China is shown in Fig. 4A. In this method, the molasses is