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Repeated open fermentative production of optically pure L-lactic acid using a thermophilic *Bacillus* sp. strain

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ABSTRACT

A thermophilic *Bacillus* sp. strain 2–6 was used in completely open repeated batch fermentation for producing optically pure L-lactic acid. Up to 107 g l^{-1} L-lactic acid of optical purity 99.8% was obtained with NaOH as pH regulator. Unexpectedly, accumulated cells did not necessarily lead to increased L-lactic acid production. Kinetic and viable cells analyses revealed that L-lactic acid production by *Bacillus* sp. 2–6 was closely related to the growth of viable cells. Accumulated unviable cells had a slight or negative effect on production efficiency. Therefore, the number of repeated batches should be limited to avoid the accumulation of unviable cells. In this study, repeated open operation was shown to be feasible for optically pure L-lactic acid production, and new light shed on the roles of viable and unviable cells in repeated batch fermentation.

1. Introduction

Lactic acid has traditionally been used in the food and pharmaceutical industries as a preservative and drug precursor. At present, more than 90% of the raw materials for chemical industry, which are mainly converted to polymers, are derived from fossil feedstocks (Mecking, 2004). Increasing concerns about oil depletion and environmental pollution by plastics have been the driving force for identifying naturally degradable polymers derived from renewable feedstocks. In the biodegradable polymer industry, lactic acid has found new applications as a monomer of poly(lactic acid) (PLA), which is one of the most promising biodegradable plastics of the future. High optical purity is a prerequisite when lactic acid is used as a PLA monomer because poly(DL-lactide) has an amorphous form due to the random distribution of L- and D-lactide units (Nair and Laurencin, 2007).

Lactic acid fermentation has been extensively studied using various lactic acid producers such as fungi (Ganguly et al., 2007), lactic acid bacteria (John et al., 2006; Kwon et al., 2000; Passos et al., 1994; Senthuran et al., 1999), and genetically modified strains (Saitoh et al., 2005; Zhou et al., 2003). In comparison with other microorganisms, thermophilic bacteria such as *Bacillus* sp. can be used in non-sterilized fermentation (Qin et al., 2009). Open or non-sterilized fermentation strategy is favorable because (1) Maillard reaction and formation of furfural compounds during sterilization can be avoided; (2) equipment requirement and energy consumption can be lowered; and (3) fermentation process can be simplified and labor can be saved (Qin et al., 2009). In comparison with batch fermentation, repeated batch operation leads to great savings in terms of both time and labor. These include less time required for washing and sterilizing the fermentor, omission of seed preparation time, high growth rates, and short main culture time due to the high initial inoculation volume (Naritomi et al., 2002). By combining the advantages of open fermentation and repeated batch operation, a further energy-efficient, labor- and time-saving operation strategy can be established. The open repeated batch fermentation has not been reported for lactic acid production, especially for producing polymer-grade L-lactic acid. On one hand, the open repeated batch fermentation would make L-lactic acid production much easier to handle and energy-efficient. On the other hand, the strain used in this strategy must be highly robust to resist increased possibility of contamination during the repetition of open culture, especially for producing highly optically pure lactic acid.

Alkaline agents are used to maintain a neutral environment for lactic acid production. In comparison with $CaCO_3$, NaOH is a simpler and cleaner alternative. When $CaCO_3$ is used, the calcium lactate produced has to be converted to lactic acid by H_2SO_4, and the resulting by-product $CaSO_4$ is a solid waste that is produced in large amounts and has little utility. In contrast, when NaOH is used, the sodium lactate produced can be electrodialyzed to lactic acid,

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and NaOH that is produced as a by-product can be reused (Persson et al., 2001).

Lactic acid production is closely associated with cell growth and cell maintenance of lactic acid producers. High cell density achieved by cell-recycle is believed to be able to improve lactic acid production (Choudhury and Swaminathan, 2006). However, little information has been reported on the interaction of viable and unviable cells in high cell density culture and their roles for L-lactic acid production during this process.

To investigate the possibility of an energy-efficient strategy for producing optically pure L-lactic acid, open repeated batch fermentation was conducted with a thermophilic *Bacillus* sp. strain. The interaction of viable and unviable cells of the *Bacillus* sp. strain and their influence on L-lactic acid production were also studied.

2. Methods

2.1. Microorganisms and cultivation

Thermophilic *Bacillus* sp. 2–6 used in this study was isolated in our laboratory (Qin et al., 2009). Mesophilic *Lactobacillus casei* ATCC 334 was purchased from the American Type Culture Collection. The stock culture was maintained on MRS agar slants. The initial pH was adjusted to 6.2–6.5 using 10 M NaOH. The slants were incubated for 12 h and stored at 4 °C. The incubation temperatures for *Bacillus* sp. 2–6 and *L. casei* ATCC 334 were 50 and 37 °C, respectively. The stock culture was transferred to fresh MRS agar slants every 3–4 weeks.

The following medium was used for seed preparation (in g l^{-1}): glucose, 50; yeast extract, 10; and $CaCO_3$, 30. A loop of cells from the stock culture was inoculated into 50 ml of seed medium and incubated at 150 rpm for 12 h. The incubation temperatures for *Bacillus* sp. 2–6 and *L. casei* ATCC 334 were 50 and 37 °C, respectively. The media was sterilized at 121 °C for 20 min. The inoculum size was 10% (v/v).

The fermentation medium had 130 g l^{-1} glucose and different concentrations of yeast extract, as described in the experiments. Open repeated batch fermentation was conducted for nine runs. The cultivation time for each batch was approximately 48 h. In the first four runs, 10 g l^{-1} yeast extract was used to investigate the influence of batch repetition on L-lactic acid production. In batches 5–8, nutrient requirement was studied by using yeast extract at 2, 10, 15, and 20 g l^{-1}, respectively. In batch 9, the initial cell density was set at 36 (OD_{620}), and 10 g l^{-1} yeast extract was used to test whether the same nutrient used in the first four batches could support the growth of higher initial cell density. To compare the optical purity of L-lactic acid produced by *Bacillus* sp. 2–6 and *L. casei* ATCC 334, open repeated fermentation was conducted for seven batches. To calculate the viable cells of *Bacillus* sp. 2–6, open batch fermentation was cycled for 10 times. Fermentation broth was sampled at the beginning of each batch for viable cell analysis. Glucose (45 g l^{-1}) and yeast extract (10 g l^{-1}) were the components of the fermentation medium for viable cell analysis of *Bacillus* sp. 2–6, and the open culture was repeated every 12 h.

A 5-l fermentor (Biostat B., B. Braun Biotech International GmbH, Melsungen, Germany) with a working volume of 3 l was used for open repeated batch fermentation in this study. The culture pH was automatically maintained at 6.5 by adding 10 M NaOH. The fermentation temperatures for *Bacillus* sp. 2–6 and *L. casei* ATCC 334 were controlled at 50 and 37 °C, respectively. The agitation rate was set at 200 rpm. At the end of each batch, the broth was centrifuged at 3399*g* for 15 min. All recycled cells were used as the seed for the next batch. All batch fermentations were conducted openly (without sterilization).

2.2. Analytical methods

The glucose and L-lactic acid levels were measured using an SBA-40C biosensor analyzer (Institute of Biology, Shandong Province Academy of Sciences, PR China). Cell density was measured spectrophotometrically at 620 nm. The number of viable cells was determined in terms of colony-forming units (CFU). The optical purity of L-lactic acid was determined using a high-performance liquid chromatography (HPLC) system (Agilent 1100 series, Hewlett–Packard, USA). A chiral column (MCI GEL CRS10W, Japan) was used with 2 mM $CuSO_4$ as the mobile phase at 0.5 ml min^{-1} (25 °C). The UV detector was set at 254 nm. The optical purity of L-lactic acid was defined as follows:

$$\text{optical purity} = \frac{\text{L-lactic acid}}{\text{L-lactic acid} + \text{D-lactic acid}} \times 100\%$$

3. Results and discussion

3.1. Comparison of the optical purity of L-lactic acid by strain 2–6 and *L. casei* ATCC 334

The optical purity of L-lactic acid produced by *Bacillus* sp. 2–6 and *L. casei* ATCC 334 was compared, and the results are shown in Fig. 1. During completely open repeated batch fermentation, *Bacillus* sp. 2–6 produced L-lactic acid of optical purity higher than 99% in all batches. In contrast, L-lactic acid produced by *L. casei* ATCC 334 had optical purity from 96.1% to 98.5%, which was much lower than that of L-lactic acid produced by *Bacillus* sp. 2–6. In the second batch, the optical purity dramatically decreased to almost 96.1%. Although the optical purity generally increased in subsequent cycles and reached 98.5% in the final batch, it remained below that of *Bacillus* sp. 2–6 in all the batches. These findings indicated that in comparison with mesophilic L-lactic acid producer, thermophilic *Bacillus* sp. 2–6 is able to produce L-lactic acid of high optical purity. Moreover, the high optical purity level was maintained in successive cycles of open repeated fermentation. Therefore, thermophilic *Bacillus* sp. 2–6 is believed to be a more robust L-lactic acid-producing strain in comparison with mesophilic L-lactic acid producer for open repeated production of polymer-grade L-lactic acid.

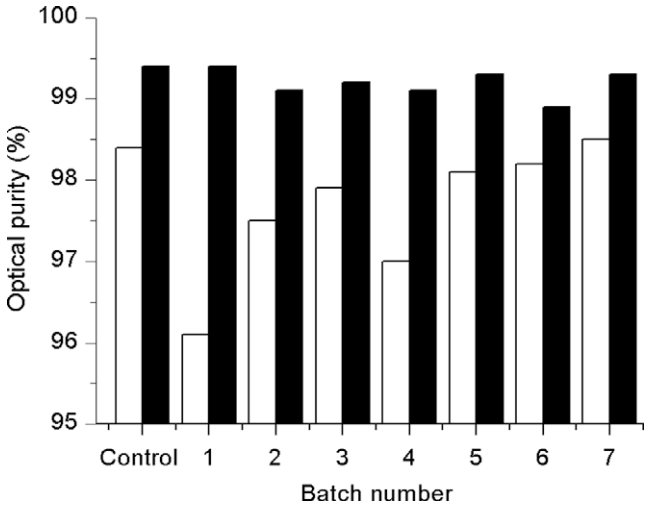

Fig. 1. Comparison of the optical purity of L-lactic acid produced by *Bacillus* sp. 2–6 (black column) and *L. casei* ATCC 334 (empty column). The controls were the strains' seed culture.