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ORIGINAL ARTICLE

Enhanced production of butyric acid through immobilization of *Clostridium tyrobutyricum* in a novel inner disc-shaped matrix bioreactor

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Abstract Cell immobilization coupled with the fermentation process and subsequent separation procedure has a great potential to improve the tolerance and efficiency of microorganisms, simplify the production process, and reduce production cost. A novel inner disc-shaped matrix (IDSM), composed of a stainless steel wire mesh and covered with fabric in a disc-shaped configuration, has been developed for butyric acid production by *Clostridium tyrobutyricum*. Our systematic investigation of the effects of fibrous materials, matrix size, and quantity on cell loading and butyric acid production resulted in a system, namely, fed-batch mode in an IDSMB, which achieved a high butyric acid concentration of 61.87 g/L, a productivity of 0.72 g/L·h productivity, and a yield of 0.42 g/g. The system was also evaluated for its long-term performance in a repeated-batch mode for 10 cycles. The highest concentration of butyric acid reached in 1 cycle was approximately 25.5 g/L, and the butyric yield varied from 0.41 to 0.47 g/g, with an average yield of 0.44 g/g per cycle. The volumetric productivity of butyric acid varied from 0.91 to 1.31 g/L·h, with an average of 1.16 g/L·h. The IDSMB system developed in this work for butyric acid production could be extremely applicable for industrial applications in organic acid biosynthesis.

Keywords Inner disc-shaped matrix · *Clostridium tyrobutyricum* · Butyric acid · Cell immobilization

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Introduction

Butyric acid is a four-carbon short chain fatty acid (Fig. 1) with many applications in different industries, such as the food processing (Armstrong and Yamazaki 1986), chemical (El-Shafee et al. 2001; Cao et al. 2011), and bio-fuel industries (Mosier et al. 2005; Li et al. 2014). The industrial production of butyric acid is mainly via chemical synthesis in a process which involves the oxidation of butyraldehyde, a substrate obtained from propylene that is derived from crude oil by oxosynthesis (Cascone 2008). At the present time, the chemical synthesis of butyric acid is preferred due to the low production cost and the abundance of starting material from petroleum. However, the continuous demand for petroleum and increasing petroleum prices, as well as the world's growing requirement for clean energy sources, have driven researchers to search for alternative methodology. One such potential alternative to complement the traditional petroleum-based chemical synthesis is the microbial production of butyric acid via fermentation methodology, which is considered to be environmental friendly and involves the use of bio-based products. Most butyric acid-producing microbial strains are members of the genus *Clostridium*, including *Clostridium tyrobutyricum*, *C. butyricum*, *C. beijerinckii*, and *C. barkeri* (Harris et al. 1986; Alam et al. 1988; Vandak et al. 1995; Zhu and Yang 2004; He et al. 2005). Compared to other butyrate-producing strains, *C. tyrobutyricum* is a hyper-butyrate-producing and obligate anaerobic strain with a higher

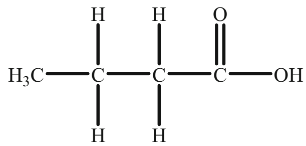

Fig. 1 Structure of butyric acid

tolerance to hash conditions which can produce butyric acid with a higher yield, purity, and stability. As such, it exhibits a good potential for industrial application.

Conventional free-cell fermentation of *C. tyrobutyricum* for butyric acid production is characterized by low productivity, low concentrtion of the final product, and low yield. Many effective strategies have been developed to overcome these problems, including improvements in strains and fermentation technology as well as in downstream process technology, such as modifying genes of microbial strains (Zhu et al. 2005; Liu et al. 2006), choosing suitable fermentation modes (Michel-Savin et al. 1990a), controlling the feeding mode of the supportive medium (Fayolle et al. 1990; Michel-Savin et al. 1990b; Zhu et al. 2002), and designing a suitable immobilized cell bioreactor (Huang et al. 2002). A new fibrous bioreactor containing a packed bed of fibrous matrix with immobilized cells has recently been successfully developed and used to produce butyric acid and several other organic acids (acetic acid, propionic acid, lactic acid), with significant improvements in productivity and yield (Huang et al. 1998; Tay and Yang 2002; Suwannakham and Yang 2005; Huang et al. 2011). Likewise, a rotating-disc bioreactor has also been widely used in the waste water treatment processes for its simplicity to operate and convenience to scale up, with strains immobilized on the surface of the discs through physical adsorption. The success of both the fibrous-bed bioreactor and rotating-disc bioreactor inspired the development of a novel inner disc-shaped immobilization matrix for butyric acid fermentation, with the aim to to improve the mass transfer efficiency of the bioreactor and the utilization rate of the fermentation equipment, as well as reduce the risk of bacterial contamination. This system can be easily assembled for scalable application.

In this study, we evaluated the feasibility and efficiency of this novel inner disc-shaped matrix with *C. tyrobutyricum* cells immonbilized on the discs to enhance the production of butyric acid and also investigated the performance and stability of the immobilized cells for long-term production of butyric acid. The effects of fibrous materials on the immobilized biomass, butyric acid production, and cell relative viability were studied in anaerobic serum bottles, and the effects of matrix size and quantity on biomass immobilization and butyric acid production were systematically tested by batch fermentation in a 5-L stirred-tank bioreactor. Finally, the immobilized matrix in a stirred-tank bioreactor was operated in both fed-batch and repeated-batch fermentation modes to evaluate the stability of long-term production of butyric acid. The fermentative kinetics, including final product concentration, butyric acid yield, and volumetric productivity, were investigated. Our results show that efficient production of butyric acid in this novel inner disc-shaped matrix bioreactor (IDSMB) is feasible.

Materials and methods

Culture and media

A hyper-butyric acid-producing strain *Clostridium tyrobutyricum* ZJU 8235, derived from the strain adaption in a fibrous bed bioreactor, was provided by Professor Zhinan Xu from Zhejiang University. The stock culture was kept in anaerobic serum bottles containing the semi-synthetic medium described previously (Huang et al. 2011). For seed culture, 2 mL strain stock was first inoculated into an anaerobic serum bottle containing 50 mL growth medium and incubated at 37 °C for about 12 h, when the optical density value at 600 nm (OD_{600}) reached 6.0, then 100 mL of this seed suspension was transformed into a 5-L stirred-tank bioreactor containing 3 L fermentation medium. Cell immobilization was carried out by stirring the fermentation broth through the fibrous bed. After about 1 day of cultivation, the fermentation broth in the fermentor was replaced with fresh medium to start a new batch. Unless otherwise noted, the seed suspension medium contained 30 g/L glucose, 5 g/L yeast extract (Bio Basic Inc., China), 5 g/L peptone (Bio Basic Inc.), 3 g/L $(NH_4)_2SO_4$, 1.5 g/L K_2HPO_4, 0.6 g/L $MgSO_4{\cdot}7H_2O$, 0.03 g/L $FeSO_4{\cdot}7H_2O$, 0.3 g/L L-cysteine hydrochloride anhydrous, and 0.05 mL resazurin solution (1 %, w/v). The fermentation medium contained 60 g/L glucose, 5 g/L yeast extract (Bio Basic Inc.), 5 g/L peptone (Bio Basic Inc.), and the same concentrations of mineral salts as the seed suspension medium. All media were sterilized at 121 °C for 30 min.

Effects of fibrous materials on cell loading and relative viability

To investigate the effects of fibrous materials on cell loading and relative viability, we separately tested six fabrics, including natural fabrics (cotton, linen, bamboo, wool) and synthetic fabrics (polyester, acrylic), in 100 mL anaerobic serum bottles. Each anaerobic serum bottle contained a different fabric (2×2 cm; the dry weight was measured before use) and 60 mL fermentation medium. After culturing at 37 °C for 48 h, the dry weight of the immobilized biomass on the different fabrics was measured. For comparison, an anaerobic serum bottle without immobilization fabric was also investigated under the same culture condition. The relative viabilities of the cells were measured by the TTC (2,3,5-triphenyl-2H-tetrazolium chloride) method (Suwannakham and Yang 2005). Briefly, the immobilization cells on each piece of fabric were eluted by 0.1 mol/L potassium phosphate buffer solution and suspended in phosphate buffer solution (free cells were eluted and collected directly). TTC was added to the cell suspension to give a final concentration of 0.1 % (w/v), then incubated for 30 min at room temperature and centrifuged at 10,000 rpm and 4 °C for 10 min. After the addition of the same volume of ethanol, the supernatant was collected via centrifugation. The absorbance (A_1) of the supernatant at 485 nm was measured on an ultraviolet spectrophotometer using a pure ethanol solution as the blank standard. We considered 1 mg of active cells to correspond to an absorbance of 0.242. The cells cultured in serum bottles in the stationary phase were referred to as the control with 100 %

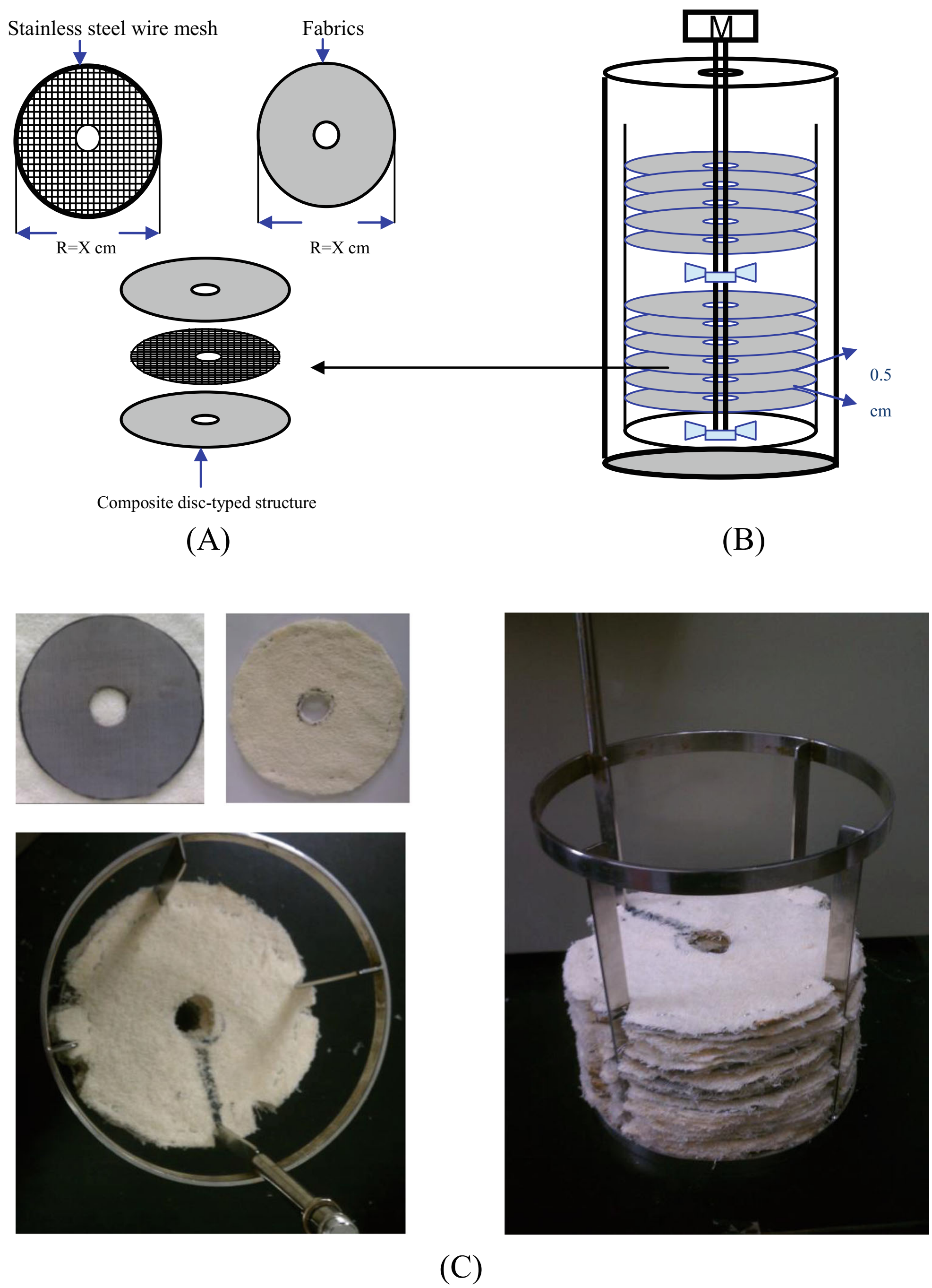

Fig. 2 Schematic of the inner disc-shaped matrix bioreactor (IDSMB). The inner disc-shaped immobilization matrix consisted of a stainless steel wire mesh covered with fabric (**a**). Matrices (interval between each matrix 0.5 cm) were fixed in a 5-L stirred-tank bioreactor (diameter 17.5 cm) (**b**). **c** Picture of the actual inner disc-shaped matrix

Table 1 Comparison of the relative viability between immobilized cells and free cells with different fibrous materials in 100 mL anaerobic serum bottles

Immobilization material	Relative viability (%)		Cell loading (mg DCW/g fiber)	Butyrate (g/L)
	Free cells	Immobilized cells		
Linen	38±1.4	69±2.4	33.29±1.86	6.34±0.12
Cotton	40±2.3	73±3.2	40.77±2.74	8.67±0.21
Bamboo	43±1.9	71±2.1	24.94±1.05	7.13±0.15
Wool	41±3.2	67±2.9	35.23±2.36	8.06±0.23
Polyester	34±2.8	60±1.5	24.64±1.53	5.65±0.08
Acrylic	31±1.7	58±1.5	32.29±1.01	6.27±0.14

DCW, Dry cell weight

Data are presented as the mean ±standard deviation (SD) (n=3)

viability (A_2). The relative viability of immobilization cells was formulated as follow:

$$\text{Relative viability } = A_1 \Big/ A_2 \times 100\ (\%)$$

Fermention kinetics in the inner disc-shaped matrix bioreactor

As shown in Fig. 2, the inner disc-shaped immobilization matrix consisted of a stainless steel wire mesh covered with the chosen fabric. Disc-shaped matrices of four different sizes (matrix diameter 10, 12, 14, 16 cm; interval between each matrix 0.5 cm) were investigated to study their effects on cell immobilization and butyrate acid production in a 5-L stirred-tank bioreactor (B. Braun Biotech international, Germany; diameter of 5-L stirred-tank bioreactor 17.5 cm). All matrices were sterilized twice by autoclaving at 121 °C for 30 min before use.

The butyric acid production process was carried out in a 5-L stirred-tank bioreactor containing 3 L fermentation medium. Different fermentation modes, namely, batch, fed-batch, and repeated-batch modes, were individually tested in the inner disc-shaped matrix bioreactor (IDSMB) for enhancement of the yield and productivity of butyric acid with a suitable size and quantity of immobilization matrix. The cultivation conditions have been described elsewhere (Huang et al. 2010). For the fed-batch mode, concentrated glucose (700 g/L) was used for pulse feeding whenever the sugar were almost exhausted in the fermentation broth. To evaluate the fermentative process, we took samples of the fermentation medium at regular intervals (6 h) for the analysis of biomass, substrate, and product concentrations. Unless otherwise noted, all experiments were performed in triplicate, and data were expressed as the mean±standard deviation (SD). One-way analysis of various was used to compare the differences between mean values. A level of probability at p<0.05 was set as statistically significant. All data analysis was performed using SPSS version

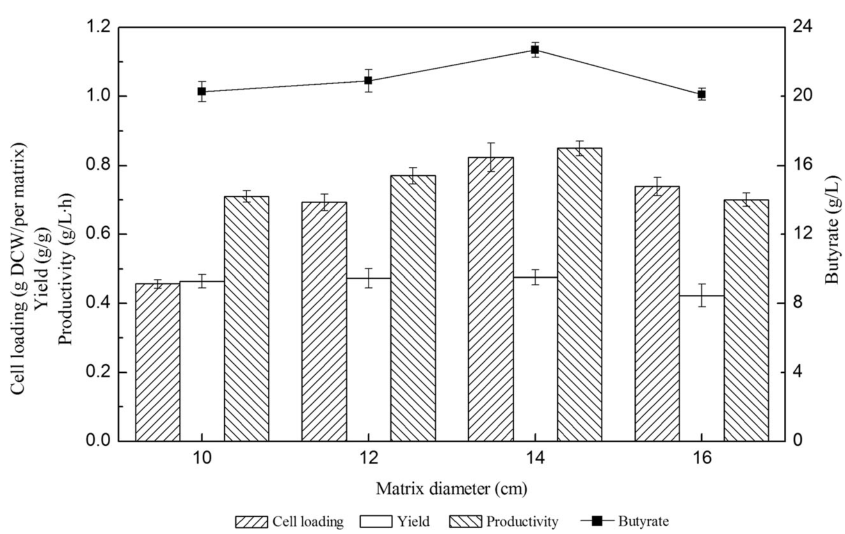

Fig. 3 Effects of the disc-shaped matrix size on cell loading and butyric acid production in a 5-L stirred-tank bioreactor (diameter 17.5 cm) at pH 6.0 and 37 °C. All trials used 12 matrices. *DCW* Dry cell weight

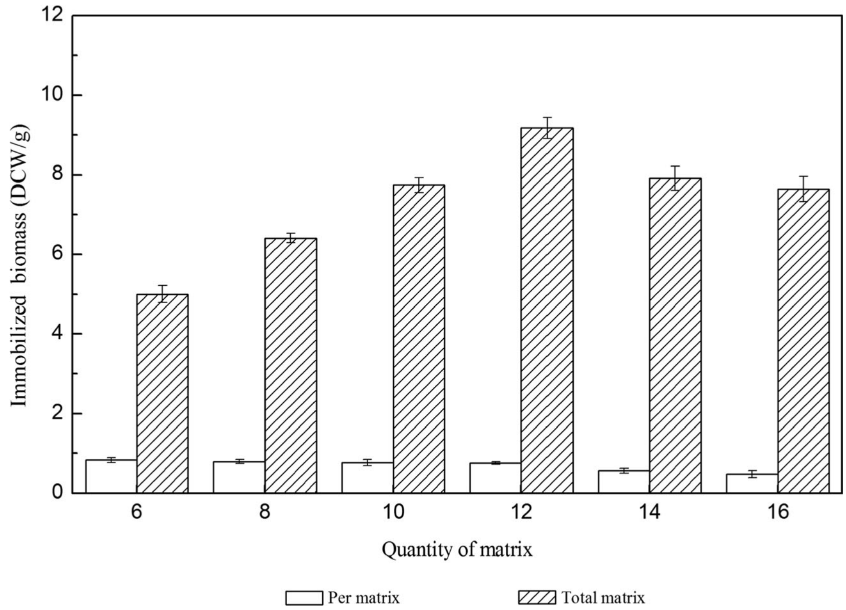

Fig. 4 Effect of the number of disc-shaped matrices on cell loading in a 5-L stirred-tank bioreactor (diameter 17.5 cm) at pH 6.0 and 37 °C. Diameter of matrices 14 cm

13.0 software for Windows (IBM SPSS Statistics, Armonk, NY, USA) .

Analytical methods

Cell growth was monitored on a spectrophotometer (model UV-16-1; Shimadzu, Japan) at a wavelength 600 nm. One unit of OD_{600} was equivalent to approximately 0.87 g/L dry cell weight (DCW). Sugars in the fermentation samples were analyzed by the 3,5-dinitrosalicylic acid colorimetry method, and the sugar content was further analyzed by high-performance liquid chromatography as described by Huang et al. (2010). The concentrations of butyric acid and acetic acid were determined using a gas chromatography system (model GC-2014; Shimadzu) equipped with a flame ionization detector and Stabilwax column (30 m×0.32 mm× 0.25 μm, d_f=0.25; model 10624; Restek Corp., USA) under the following conditions: the inlet and detector temperatures were both kept at 200 °C using nitrogen as the gas carrier; the column temperature was programmed in the range of 55–105 °C, with an initial 8 min at 55 °C, followed by a heating rate of 10 °C up to at 105 °C, and then with a heating rate of 60 °C/min to 180 °C, and 3 min at 180 °C.

Results and discussion

Effects of different fibrous materials on cell loading and relative viability

The strategy of cell immobilization has been studied for decades as a means to improve the performance and economics

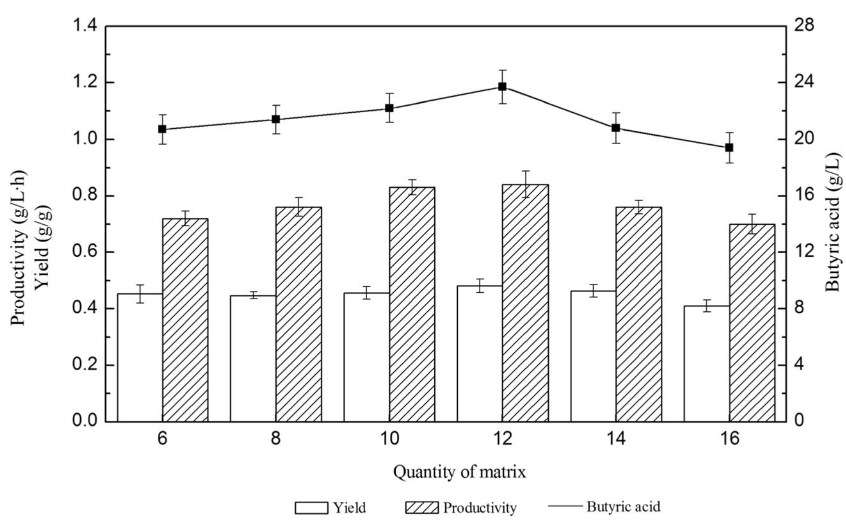

Fig. 5 Effect of the number of disc-shaped matrices on butyric acid production in a 5-L stirred-tank bioreactor (diameter 17.5 cm) at pH 6.0 and 37 °C. Diameter of matrices 14 cm

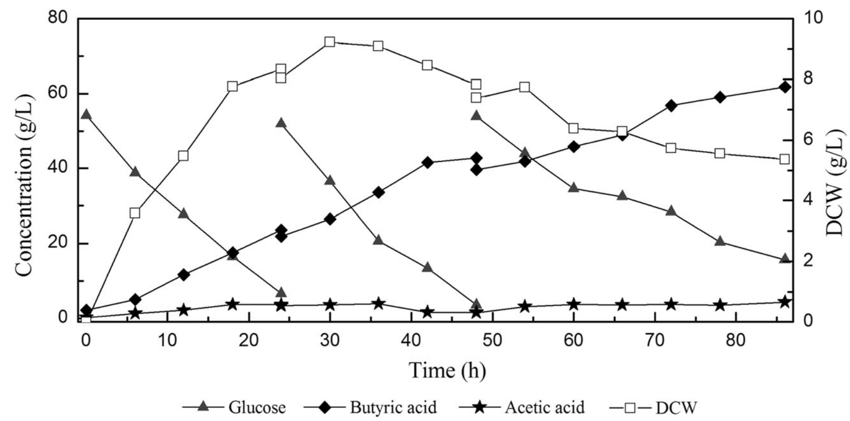

Fig. 6 Fed-batch fermentation kinetics of immobilized cells in the IDSMB at pH 6.0 and 37 °C

of many fermentation processes. Foremost, the achieved high cell density in the immobilized system could lead to an increased production efficiency. Fibrous matrices have been developed as the media for cell immobilization in fibrous bed bioreactors (FBB) due to their high surface area, high void volume, low cost, high mechanical strength, high permeability, and low pressure drop (Huang et al. 1998; Zhu et al. 2002; Suwannakham and Yang 2005).

To identify the optimal fibrous material for immobilization of *C. Tyrobutyricum* and production of butyric acid, we investigated six different fabrics (linen, cotton, bamboo, wool, polyester, acrylic) for cell loading and relative viability in 100-mL anaerobic serum bottles. As shown in Table 1, among these six fibrous materials, natural cotton showed the best capability for cell loading, with the highest adherence of cells (40.77±2.74 mg DCW/g fiber). We noted that cell loading (maximum amount of cells adsorbed) was dependent not only on the initial cell concentration, pH, ionic strength, and media composition (Kilonzo et al. 2011), but also on the fibrous material.

We also took into account the possible productivity loss after long-term operation due to the accumulation of dead cells and culture degeneration and evaluated the relative viability of the immobilized cells. As summarized in Table 1, nature cotton also exerted a good performance on cell relative viability and butyrate production. In contrast, the usage of synthetic fibers (polyester, acrylic) as immobilized fabric materials resulted in a relative decline in cell loading, cell relative viability, and butyric acid production. These results clearly demonstrate that the fabric material used for cell immobilization can affect cell loading and cell relative viability. In their scanning electron microscopy study, Jiang et al. (2011) demonstrated that the rough surface of cotton fabric, with its many folds, can increase the contact area of cells. Therefore, we used cotton fabric material in subsequent studies.

Kinetics of batch fermentation in the IDSMB

Effects of matrix size

Four different sizes of the disc-shaped matrix were evaluated in a 5-L stirred tank bioreactor (diameter 17.5 cm). As shown in Fig. 3, in general increases in matrix diameter enhanced cell loading onto the surface of the matrices and the production of butyric acid, although the highest butyrate production (22.13

Table 2 Comparison between free cell and immobilized cell batch fermentation for butyric acid production with differential operating modes

Fermentation parameter	Free-cell fermentation mode		Immobilized cell fermentation mode	
	Batch	Fed-batch	Batch	Fed-batch
Fermentation time (h)	30±2	107±2	24±2	85±2
Fermentable sugar utilized (g/L)	43.55±2.17	153.47±2.42	47.52±2.13	150.68±2.51
Fermentable sugar utilization rate (g/L·h)	1.45±0.03	1.43±0.04	1.98±0.03	1.77±0.03
Butyrate concentrations (g/L)	18.48±0.92	58.32±3.01	21.47±1.07	61.87±2.37
Butyrate yield (g/g)	0.42±0.01	0.38±0.01	0.45±0.01	0.42±0.01
Butyrate productivity (g/L·h)	0.62±0.02	0.55±0.02	0.85±0.03	0.73±0.01
Acetate concentration (g/L)	3.46±0.16	5.46±0.21	2.49±0.17	4.23±0.18
Butyrate/acetate ratio (g/g)	5.34±0.23	10.68±0.46	8.62±0.38	14.61±0.57

Data are presented as the mean±SD (*n*=3)

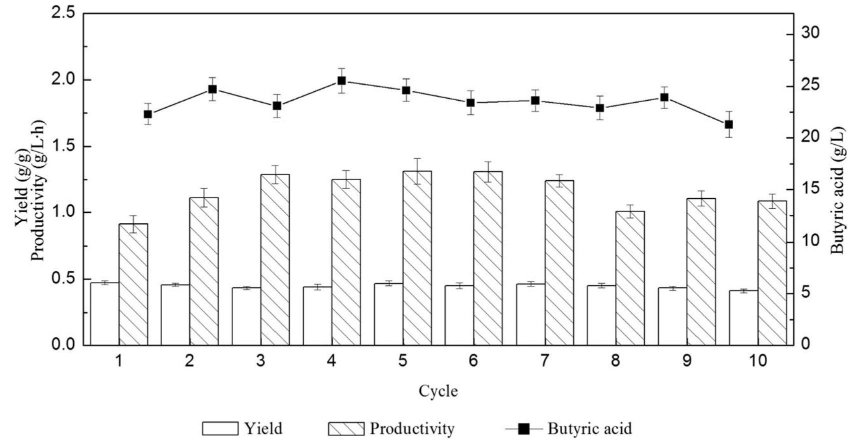

Fig. 7 Long-term production of butyrate acid by immobilized cells in the IDSMB bioreactor at pH 6.0 and 37 °C

±0.43 g/L) and productivity (0.85±0.02 g/L·h) were achieved with the 14-cm disc-shaped matrix. On the 16-cm-diameter matrix, the cells grew into large clumps, which reduced cell growth and butyric acid production because of the mass transfer limitation. In contrast, the smaller matrices (diameter 10 or 12 cm) bore fewer immobilized cells, thus also limiting butyrate production and productivity. In addition, no clumps were found on matrices with a diameter of 10, 12, or 14 cm. These results would appear to show that matrix diameter has a notable effect on the cell immobilization and butyrate production. Similar findings have also been reported for the production of lactic acid by immobilized *Rhizopus oryzae* in a honeycomb fibrous matrix (Wang et al. 2010).

Effects of matrix quantity

The effect of differing numbers of inner disc-shaped matrices on cell loading and butyrate production was also investigated. Below 12 units (=matrices), cell loading on each matrix and on all matrices together improved with increasing matrix number (Fig. 4). In contrast, when the matrix number was more than 12s, large clumps formed such that a saturated state was reached; consequently, cell loading decreased slightly. It appeared that surplus immobilized matrix can impair the mass transfer efficiency and thus limit cell growth during fermentation. In our study, butyrate concentration (23.70±1.19 g/L), yield (0.48±0.02 g/g), and productivity (0.84±0.05 g/L·h) reached their maximum values when there were 12 matrices (Fig. 5).

Kinetics of fed-batch fermentation in the IDSMB

In order to improve the production of butyric acid, we investigated the fed-batch fermentation kinetics of immobilized cells using glucose as carbon source. As shown in Fig. 6, glucose was stably consumed during the fermentation process, resulting in steadily increasing concentrations of butyric acid to the maximum level of 61.87 g/L at 86 h; the productivity and yield of butyric acid reached 0.72 g/L·h and 0.42 g/g, respectively. These results are comparable to or even better than those reported previously in a fibrous bed bioreactor where a cotton towel and a stainless-steel mesh were wound together in a spiral configuration with 3-mm gaps between each turn of the spiral layer (Huang et al. 1998, 2002; Zhu et al. 2002; Huang et al. 2011).

Compared to free-cell fermentation (Table 2), the duration of immobilized cell fermentation was greatly shortened due to the elimination of the cell lag phase (Jiang et al. 2010; Huang et al. 2011; Jiang et al. 2011), and carbon source utilization was also faster. At the end of fermentation, the concentration and yield of butyric acid obtained in the immobilized cell mode was significantly higher than that obtained in the free-

Table 3 Comparison between the inner disc-shaped matrix bioreactor and other bioreactors in terms of butyric acid production

Bioreactor	Operating mode	Butyrate (g/L)	Yield (g/g)	Productivity (g/L·h)	Reference
Fibrous bed reactor	Batch	13.7–43.2	0.35–0.46	0.57–1.23	Huang et al. 2010; Jiang et al. 2010; Huang et al. 2011)
	Fed-batch	24.9–60.4	0.29–0.38	0.33–1.14	
	Repeated-batch	10.1–27.5	0.29–0.50	0.33–3.35	
Inner disc-shaped matrix bioreactor	Batch	23.7	0.48	0.84	This study
	Fed-batch	61.9	0.42	0.72	
	Repeated-batch	21.5–25.5	0.41–0.47	0.91–1.31	

Data are presented as the mean±SD (n=3)

cell mode. The concentration was increased by 16.18 % in the batch fermentation mode and by 5.94 % in the fed-batch fermentation mode, respectively; the yield was increased by 7.14 % and 10.53 %, respectively. Additionally, compared to free-cell fermentation, immobilized cell fermentation was characterized by a higher butyrate/acetate ratio (batch: 8.62 vs. 5.34 g/g; fed-batch: 14.61 vs. 10.68 g/g), which might be due to the acceleration of cell growth rate using the cell immobilization method.

Kinetics of repeated-batch fermentation in the IDSMB

To further evaluate the long-term stability of the IDSMB, the batch fermentation was repeated for 10 cycles over a 230-h period. As shown in Fig. 7, the IDSMB performed well in terms of butyric acid production, with the highest concentration of butyric acid, approximately 25.5±1.18 g/L, achieved during the repeated-batch fermentation. At the end of the fermentation period, the variation of butyric yield in the ten batches varied from 0.41±0.01 to 0.47±0.01 g/g, with an average of 0.44 g/g. The volumetric productivity of butyric acid in each batch varied from 0.91±0.06 to 1.31±0.09 g/L·h, with an average of 1.16 g/L·h. Compared with the fermentation processes using the fibrous bed bioreactor (Huang et al. 2010; Jiang et al. 2010; Huang et al. 2011), the yield, productivity, and concentration of butyrate obtained with the disc-shaped matrix was a little lower (Table 3) due to inhibition by the end-product of butyrate. In terms of commercial application, the IDSMB circumvents the need for an external tank and separate sterilization and power system for broth recycling; as such, this system can be easily adapted to industrial-scale processing.

Conclusion

We have described the development of a novel inner disc-shaped matrix bioreactor that has great potential for the industrial-scale production of butyric acid with immobilizing *Clostridium tyrobutyricum*. The use of immobilized cells in this bioreactor gave a high yield of butyric acid (up to 0.47 g/g), and the process was stable for 10 continuous cycles in repeated-batch fermentation. A high butyric acid concentration of approximately 61.9 g/L and a productivity of approximately 0.73 g/L·h were achieved in the fed-batch fermentation. The IDSMB should be investigated in detail in fluid dynamics studies. Current fermentation kinetics provides an alternative strategy for the commercial production of butyric acid, as well as several other organic acids (acetic acid, propionic acid, and lactic acid), in the future.

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