African Journal of Microbiology Research Vol. 5(6) pp. 661-666, 18 March, 2011
Available online http://www.academicjournals.org/ajmr
DOI: 10.5897/AJMR10.596
ISSN 1996-0808 ©2011 Academic Journals

Full Length Research Paper

Continuous butyric acid production by corn stalk immobilized *Clostridium thermobutyricum* cells

Wei Li[1], Hui-Juan Han[2] and Chun-hui Zhang[3]*

[1]College of Food Science and Engineering, Ocean University of China, Qingdao 266003, China.
[2]College of Chemistry and Environmental Science, Henan Normal University, Xinxiang, 453007, China.
[3]Henan Sansheng Pharmaceutical Industrial Co. Ltd., Xinxiang 453000, China.

Accepted 1 March, 2011

Corn stalks were used as a support to immobilize *Clostridium thermobutyricum* in a fermentation process for butyric acid production. The effects of pH and acetic acid concentration on butyric acid production were examined in a steady-state 25-day continuous flow operation. A metabolic shift was induced by changing pH of the medium. The maximum and the minimum ratio of butyric acid and acetic acid produced in the fermentation process were achieved at pH 7.0 and 5.0, respectively, and the maximum yield of butyric acid was observed at pH 6.0. The addition of acetate in the medium has resulted in an increased butyric acid final concentration. The maximum total butyric acid concentration of 15.82 g/l was obtained at pH 6.0 with 10 g/l acetic acid concentration in the medium. The cell adsorption and morphology change during the growth phase on corn stalk support were examined by the scanning electronic microscope (SEM).

Key words: Butyric acid, corn stalk, immobilized cells, *Clostridium thermobutyricum.*

INTRODUCTION

Butyric acid, a 4-carbon short chain fatty acid, is widely used in the chemical, food and pharmaceutical industries (Zigová and Šturdík, 2000; Zhu and Yang, 2004; Hijova and Chmelarova, 2007). Today, butyric acid production is dominated by chemical synthesis in industry with starting materials derived from crude oil (Zhu and Yang, 2004). However, with the decreasing supply of world crude oil, the increasing amounts of food industry by-products, and the increasing consumer demand for organic natural products in food additives, pharmaceutical products, and preservatives, the production of butyric acid by microbial fermentation is becoming economically attractive (Zigová and Šturdík, 2000; Hijova and Chmelarova, 2007). At present, the contradiction between low production and high demands is becoming acute, giving rise to the urgency of addressing the problem of increasing the production of butyric acid. Thus fermentation process for butyric acid production can play an important role in the supply chain (Zhang et al., 2009a).

Recent study has shown that there are many advantages of using thermophilic microorganisms for organic acids production through traditional fermentation (Demain et al., 2005). Thermophiles were believed to impart several significant advantages, such as increased production rates, improved mass transfer, and reduced susceptibility to contamination. Among thermophilic clostridia, only *Clostridium thermobutyricum* produced high ratio butyric acid at 55°, which is close to the temperature of commercial cellulose enzymatic hydrolysis process. This clostridium may be a potential strain for butyrate production in the future if its characteristics are sufficiently advantageous (Wiegel et al., 1989; Canganella and Wiegel, 2000; Canganella et al., 2002; Zhang et al., 2009a).

There are several reasons preventing the use of fermentation process for butyrate production at commercial scale compared to that of petrochemical routes. Those include low productivity, low yield, and the high cost of product recovery (Zigová and Šturdík, 2000). A continuous culture system with immobilized cells is a promising technique to improve butyric acid productivity

*Corresponding author. E-mail: zhmy26@126.com. Tel: +86+373+3329121.

and to reduce product separation cost (Mitchell et al., 2009). There are many techniques for cell immobilization, among these a fibrous-bed bioreactor (FBB) with cells immobilized in the fibrous matrix packed in the reactor has been successfully used to produce several of the most commercially used organic acids (Huang et al., 2002; Jiang et al., 2009).

One of the most extensively used classes of natural support for cell immobilization is lignocellulosic materials. Corn stalks are a readily available and inexpensive material with many advantages as a supporting material for cell immobilization. These advantages include being highly porous as well as having a good water retention capacity. One of the most attractive properties of using cellulosic materials as an immobilization support is that the spent materials can be sent back to the hydrolysis process for sugar production, thus minimizing waste generation (Zhang et al., 2009b). This paper describes the feasibility of using corn stalks as a support and delineates a novel and simple technique for quick cell loading while providing a strong support for immobilized cells. The effects of acetic acid concentration and culture pH on butyric acid production are also discussed in this paper.

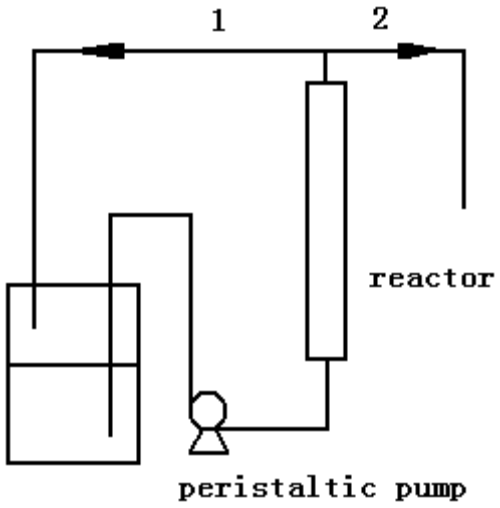

Figure 1. A diagram of the immobilized cell reactor. When the reactor was circulated with growth media for cell immobilization the effluent was going through pipe 1 back to the media storage tank. During butyric acid production operation, the storage tank was switched to a tank filed with fermentation media, and the effluent was discharged through pipe 2.

MATERIALS AND METHODS

Strain and medium

Stock culture of *C. thermobutyricum* strain JW 171K (ATCC 49875) was obtained from ATCC (American Type Culture Collection). The organism was grown in a basal medium that included (per liter): 1.3 g of $(NH_4)_2SO_4$, 2.6 g of $MgCl_2 \cdot 6\ H_2O$, 1.43 g of KH_2PO_4, 7.2 g of $K_2HPO_4 \cdot 3\ H_2O$, 0.13 g of $CaCl_2 \cdot 2\ H_2O$, 6 g of Na-ß-glycerophosphate, 1.1 mg of $FeSO_4 \cdot 7\ H_2O$, 5 g of yeast extract, 1 mg of resazurin. The pH of the medium was adjusted to 6.8 using 2 M NaOH solution prior to sterilization. Glucose (50% w/v solution under O_2-free N_2 gas) was autoclaved separately at 121°C for 30 min and then the stock glucose solution was added to the medium with the final concentration of glucose at 10 g/l. The microbial strains were supplemented with 50% glycerol before storage at -20°C. The cultivation conditions were 55°C, pH 6.8, 200 rpm for rotary shaker, and anaerobic atmosphere with gas mixture composition of N_2:H_2:CO_2=80: 5:15. All chemicals were purchased from Sigma unless stated otherwise (Zhang et al., 2009a).

Cell immobilization and reaction system setup

The *C. thermobutyricum* cells were immobilized by the method of physical adsorption onto corn stalks. The process for cell immobilization and the operation of the immobilized cell reactor are shown in Figure 1.The corn stalks were chopped into small pieces (5–8 mm in size) with a knife. The corn stalks were then soaked with 1% (v/v) hydrochloric acid, and the slurry was placed in a 121°C water bath for 60 min. The treated stalk was then washed twice with distilled water and dried in an oven at 80°C overnight. Treated stalk (2.0-g) was then autoclaved at 121°C for 15 min and packed at room temperature into a 2-cm i.d, 20-cm long polymethylmethacrylate column that was sterilized at 121°C for 15 min prior to use. The packed column was then flushed with anaerobic gas (80% N_2, 15% CO_2, and 5% H_2) to remove the residual moisture and O_2 inside the reactor and in the pores of corn stalk. The reactor was then placed in an anaerobic chamber in which the temperature was controlled at 55°C. The slurry of culture media with actively growing cells was pumped into the reactor by a peristaltic pump through silicone tubing at a flow rate of 10 ml h^{-1}. The cells and media mixture were then circulated for 24 h for cell adsorption and growth. The storage (buffer) tank was then switched to the fermentation media tank. The fermentation media with different pH and different acetate concentration was fed continuously to the immobilized cell reactor. Samples taken from the top of column were centrifuged and the supernatants were used for glucose and organic acid content analysis. The consumed glucose was calculated by subtracting the feed concentration from the effluent concentration of glucose.

Sample analysis

Effluent samples of 2.0 ml were withdrawn every 12 h from the top of the packed reactor. The samples were centrifuged for 10 min at 10,000 g, 4°C to separate the cells. The supernatants were stored at -20°C prior to analysis. Acetic acid, lactic acid, butyric acid, and glucose in samples were analyzed by high performance liquid chromatography (HPLC) with a Bio-Rad HPX-87H column. The eluent for HPLC was 10 mM H_2SO_4 at a flow rate at 0.6 ml/min. The auto-sampler was set to inject the sample volume at 15 µl and the total running time was set at 25 min. The AUX RANGE parameter of RI detector was set at 2. Peak height was used to calculate the concentration of each component based on calibration curve obtained with results of the standard mixture containing all the compounds at 1 g/l (Zhang et al., 2009a).

Scanning electron microscopy

For scanning electron microscopy, the samples were fixed with

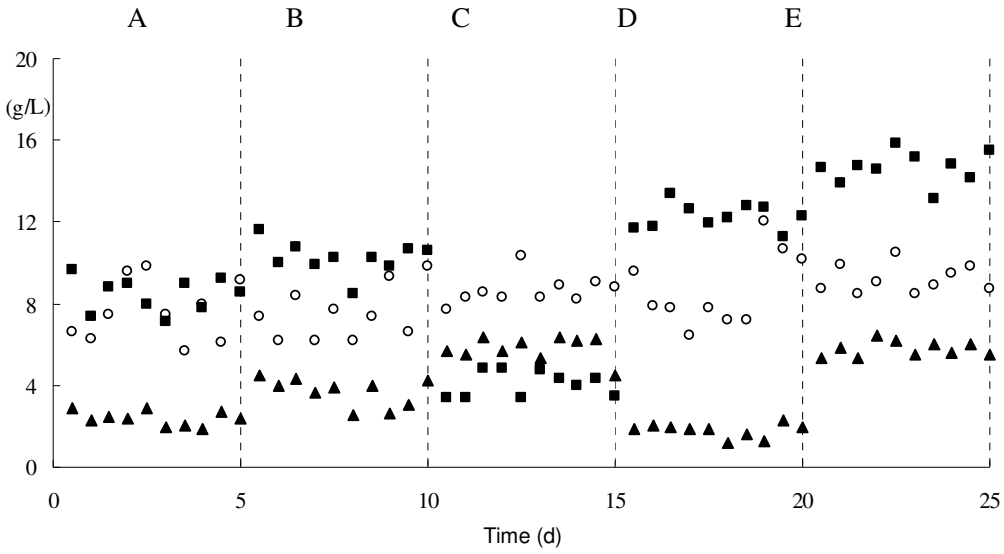

Figure 2. Effects of pH and acetic acid on butyric acid production by immobilized *Clostridium thermobutyricum* ATCC 49875 cells. butyric acid (■), acetic acid (▲),residual glucose (○).A-pH7.0, B-pH6.0, C-pH5.0, D-pH6.0+5g/l acetate, E-pH6.0+10g/l acetate.

3.5% (w/v) glutaraldehyde at 4℃ for 15 h and then washed twice with distilled water followed by a progressive dehydration with 20–100% ethanol at 20% increment. The samples were then treated with hexamethyldisilazene (HMDS) and sputter-coated with gold-palladium. The samples were scanned and photographed with a scanning electron microscope (KYKY Technology Development Ltd., China) at 25 kV (Zhang et al., 2009b).

RESULTS AND DISCUSSION

The performance of the immobilized cell reactor was investigated at various media pH, and at various acetic acid concentrations in the media when initial pH of the media was 6.0. Starting fermentation medium with 30 g/l glucose was set at pH 7.0, pH 6.0, pH 5.0, pH 6.0 + 5.0 g/l acetic acid, or pH 6.0 + 10 g/l acetic acid. Each medium composition was used and maintained for 5 days with the fluid dilution rate of 0.25 h^{-1} in continuous flow operation before switching to a different composition.

Effect of pH on butyric acid and acetic acid production in fermentation

The yield for both butyric acid and acetic acid versus fermentation time with various initial culture media pH is shown in Figure 2. Each cultural medium composition was used and maintained for 5 days and then shifted to new composition. As indicated in the Figure 2, the product concentrations were significantly affected by culture media pH. In general, butyrate production increased and acetate decreased with the pH close to 6.0. A maximum butyrate concentration of 11.65 g/l was produced at pH 6.0, with only 4.48 g/l of acetate as the byproduct (Figure 2). In contrast, acetate production increased dramatically as the pH decreased to 5.0. When pH dropped to 5.0, acetate became the major product and only a small amount of butyrate was produced in the fermentation process, indicating a clear metabolic shift from butyrate-forming to acetate- and lactate-forming pathways. It appeared that at pH 6 and higher, acetate production was inhibited. In contrast, when the pH was reduced to 5.0, acetate instead of butyrate became the major fermentation product. H_2 and CO_2 were also produced as byproducts in these fermentations.

Figure 3 illustrates the effect of pH for butyric acid fermentation. It was clear that a higher pH favors butyrate production and gives higher butyrate/acetate ratio, butyrate yield, productivity, and final concentration. The ratio of butyrate to acetate was 3.59 at pH 7.0, but decreased to 0.71 at pH 5.0. It is noted that the total organic acid production and butyric acid yields obtained at each pH varied significantly, which the main product of continuous fermentation was changed from butyrate at pH 7.0 to acetate at pH 5.0. The yields of total organic acids and butyric acid were 0.49 and 0.39 at pH 7.0, 0.62 and 0.46 at pH 6.0, 0.46 and 0.19 at pH 5.0, respectively. It was obviously that pH 6.0 was the optimal pH value for both total organic acids and butyric acid production. However, the ratio of butyrate and acetate was highest when pH was 7.0, that is, the ratio is 3.59. It was concluded that lower pH inhibited butyric acid production, and improved acetic acid production. The highest yield of total organic acids reached 92.5% of the maximum theoretical value (0.67 g/g).

The experimental data clearly showed a dramatic

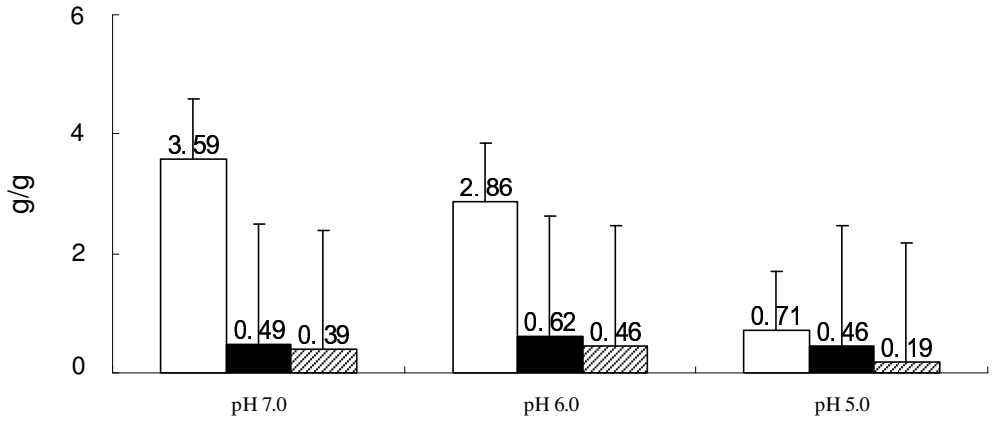

Figure 3. Effect of pH on metabolic pathway shift in fermentation. The ratio (*hollow rectangle*), the yield of total organic acids (*filled rectangle*), the yield of butyric acid (*diagonal cross*).

metabolic shift from acetate production to butyrate production with pH increase from 5.0 to 7.0. The metabolic pathway shift could be attributed to the regulation of redox potential or NADH and NAD^+, or *C. thermobutyricum* has a pathway that acetate was produced by H_2 and CO_2 utilization (Zhu and Yang, 2004).

Effect of acetic acid on butyric acid production

Acetic acid improved butyric acid production significantly (Figure 2). When 5 g/l acetic acid was added, the average concentration of butyric acid was 12.27 g/l, increased from 10.23 g/l, which was a 20% increase. The butyric acid concentration of 14.66 g/l was produced when pH was 6.0 with the addition of 10g/l acetic acid.

With increasing added acetate concentration, the production of butyrate has increased (Figure 2). The addition of 5 to 10 g/l acetate to the medium increased the final butyrate concentration to 12.27 and 14.66 g/l, respectively, indicating the re-utilization of acetate. The highest concentration of butyrate was observed at 15.82 g/l under the condition of culture media pH at 6.0 with 10 g/l acetic acid addition. However, the stoichiometric ratio of acetate consumption and butyrate formation was not consistent. When 5 g/l acetic acid was added, it was consumed at amount of 3.2 g/l and glucose was consumed at amount of 21.32 g/l; when 10 g/l acetic acid was added, and the corresponding values of consumed acetic acid and glucose concentration were 4.2 and 20.78 g/l, respectively.

Several butyrate-producing clostridia, including *C. thermobutyricum*, produce butyrate concomitantly with acetate. Acetate was the main product especially during the cell growth phase. At the end of the exponential growth, a major metabolic switch took place in *C. thermobutyricum*. The organism slowed down acetate production, and took up excreted acetate and converts it into butyrate (van Andel et al., 1985; Michel-Savin et al., 1990; Canganella et al., 2002). The purpose of recycling in the organism may be related to detoxifying the medium by reducing total hydrogen ion concentration, which was realized when one butyrate substituted for two acetates, so the metabolism shifted from more energy producing formation acetate (Glucose→ 2 acetate + $4H_2$ + 2 CO_2 + 4 ATP) to less H_2-producing butyrate formation (Glucose→ butyrate+ $2H_2$ + 2 CO_2 + 3 ATP). The addition of acetate in the medium increased the final butyrate concentration (Canganella et al., 2002; Zhang et al., 2009a). As seen in Figure 2, acetate in the medium was absorbed, and affected the final butyrate concentration, which resulted in the highest productivity of 3.955 g l^{-1} h^{-1} when 10 g/l acetic acid were in the medium.

SEM observation of the immobilized cells

Many studies (Huang et al., 2004; Jiang et al., 2009; Iqbal, 2005; Chen et al., 2009) have found that clostridium can attach to the lignocellulosic material and grow on the support without any additional chemical as nutrient. The porosity of corn stalk makes the cells not only grow on the surface of the support but also in the inner side of the porous surface of supports, resulting in a much higher cell density or cell loading per mass base. The corn stalk was treated with diluted hydrochloric acid aimed to activate the surface by removing the wax and destroying the hemicellulose. The treatment has also made the support more porous and increased surface area. Figure 4A and B showed the adherence of the cells at the end of cell adsorption operation.

The immobilized cells with significant morphological changes were observed by SEM after continuous culture for 600 h. Specifically, filamentous cells with more than 50 μm in length have appeared in the reactor. These

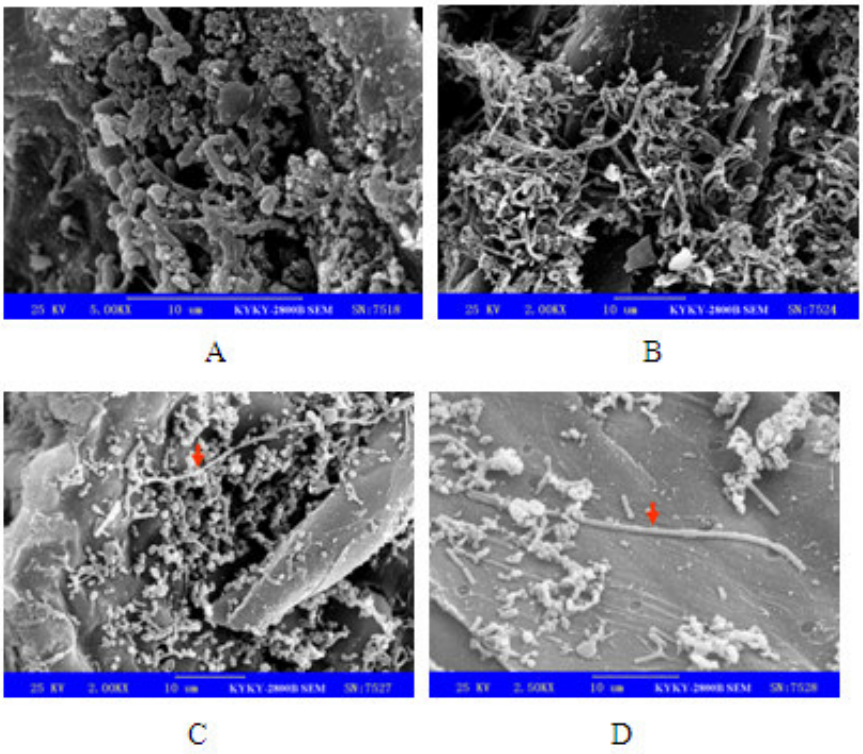

Figure 4. Scanning electron micrographs of corn stalk with immobilized *C. thermobutyricum* ATCC 49875. A. The adsorbed cells adhere to the surface of the corn stalk at the beginning of the experiment. B. A large amount of cells has been observed at the end of the experiment. C and D. Morphological changes of the immobilized cells (red arrow).

forms were usually not observed in stationary growth phases using batch cultures. The cells growing on the fibers were usually not in long filaments shape though they have been occasionally observed. The similar phenomena have been observed in some previous reported studies (Canganella and Wiegel, 2000).

It is worth pointing out that the reactor suffered from a severe gas-hold problem around the immobilization support due to CO_2 and H_2 generation by the anaerobic microbe during fermentation. The bubbles adhering to the corn stalk and gathering at the top of the reactor caused severe mass transfer inhibition and also made difficulties for steady dilution rate control. These bubbles were also difficult to be removed from the reactor.

Conclusions

The results of this study have clearly indicated that pretreated corn stalk was an effective material to immobilize *C. thermobutyricum* for butyric acid production. The maximum butyric acid productivity was achieved with culture media pH at 6.0 with 10 g/l acetic acid addition. The highest organic acids yield at 0.62 g g^{-1} and butyric acid yield at 0.46 g g^{-1} were both achieved at pH 6.0, while the maximal butyrate/acetate ratio was achieved when pH was 7.0. The pH value and acetic acid concentration have significant effects on the total organic acids and butyric acid production, productivity, and yield. The reactor was operated at steady state for 25 days in a continuous flow mode.

REFERENCES

Canganella F, Kuk SU, Morgan H, Wiegel J (2002). Clostridium thermobutyricum: growth studies and stimulation of butyrate formation by acetate supplementation. Microb. Res., 157: 149-156.

Canganella F, Wiegel J (2000). Continuous cultivation of Clostridium thermobutyricum in a rotary fermentor system. J. Ind. Microbiol. Biotechnol., 24: 7-13.

Chen F, Feng X, Li S, Oyang P, Xu H (2009). Application of bagasse as immobilization material in immobilized fiber bed reactor for producing organic acid, such as propanoic acid, lactic acid, butyric acid, and succinic acid, by fermentation. Univ. Nanjing Polytechnic, 59: 656-663.

Demain AL, Newcomb M, Wu JHD (2005). Cellulase, clostridia, and ethanol. Microbiol. Mol. Biol. Rev., 69: 124-154.

Hijova E, Chmelarova A (2007). Short chain fatty acids and colonic health. *Brat. Med. J.-Bratislavske Lekarske Listy,* 108: 354-358.

Huang WC, Ramey DE, Yang ST (2004). Continuous production of butanol by Clostridium acetobutylicum immobilized in a fibrous bed bioreactor. Appl. Biochem. Biotechnol., 113: 887-898.

Huang YL, Wu Z, Zhang L, Ming Cheung C, Yang ST (2002). Production

of carboxylic acids from hydrolyzed corn meal by immobilized cell fermentation in a fibrous-bed bioreactor. Bioresour. Technol., 82: 51-59.
Jiang L, Wang J, Liang S, Wang X, Cen P, Xu Z (2009). Butyric acid fermentation in a fibrous bed bioreactor with immobilized Clostridium tyrobutyricum from cane molasses. Bioresour. Technol., 100: 3403-3409.
M Iqbal AS (2005). Novel method for cell immobilization and its application for production of organic acid. Lett. Appl. Microbiol., 40: 178-182.
Michel-Savin D, Marchal R, Vandecasteele JP (1990). Control of the selectivity of butyric acid production and improvement of fermentation performance with Clostridium tyrobutyricum. Appl. Microbiol. Biotechnol., 32: 387-392.
Mitchell RJ, Kim JS, Jeon BS, Sang BI (2009). Continuous hydrogen and butyric acid fermentation by immobilized Clostridium tyrobutyricum ATCC 25755: Effects of the glucose concentration and hydraulic retention time. Bioresour. Technol., 100: 5352-5355.
van Andel J, Zoutberg G, Crabbendam P, Breure A (1985). Glucose fermentation by Clostridium butyricum grown under a self generated gas atmosphere in chemostat culture. Appl. Microbiol. Biotechnol., 23: 21-26.
Wiegel J, Kuk SU, Kohring GW (1989). Clostridium thermobutyricum sp. nov., a moderate thermophile isolated from a cellulolytic culture, that produces butyrate as the major product. Int. J. Syst. Bacteriol., 39: 199-204.
Zhang CH, Ma YJ, Yang FX, Liu W, Zhang YD (2009a). Optimization of medium composition for butyric acid production by Clostridium thermobutyricum using response surface methodology. Bioresour. Technol., 100: 4284-4288.
Zhang Y, Ma Y, Yang F, Zhang C (2009b). Continuous acetone–butanol–ethanol production by corn stalk immobilized cells. J. Ind. Microbiol. Biotechnol., 36: 1117-1121.
Zhu Y, Yang ST (2004). Effect of pH on metabolic pathway shift in fermentation of xylose by Clostridium tyrobutyricum. J. Biotechnol., 110: 143-157.
Zigová J, Šturdík E (2000). Advances in biotechnological production of butyric acid. J. Ind. Microbiol. Biotechnol., 24: 153-160.