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Production of Butyric Acid from Glucose and Xylose with Immobilized Cells of *Clostridium tyrobutyricum* in a Fibrous-bed Bioreactor

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Abstract Butyric acid has many applications in chemical, food, and pharmaceutical industries. In the present study, *Clostridium tyrobutyricum* ATCC 25755 was immobilized in a fibrous-bed bioreactor to evaluate the performance of butyrate production from glucose and xylose. The results showed that the final concentration and yield of butyric acid were 13.70 and 0.46 g g^{-1}, respectively, in batch fermentation when 30 g L^{-1} glucose was introduced into the bioreactor. Furthermore, high concentration 10.10 g L^{-1} and yield 0.40 g g^{-1} of butyric acid were obtained with 25 g L^{-1} xylose as the carbon source. The immobilized cells of *C. tyrobutyricum* ensured similar productivity and yield from repeated batch fermentation. In the fed-batch fermentation, the final concentration of butyric acid was further improved to 24.88 g L^{-1} with one suitable glucose feeding in the fibrous-bed bioreactor. *C. tyrobutyricum* immobilized in the fibrous-bed bioreactor would provide an economically viable fermentation process to convert the reducing sugars derived from plant biomass into the final bulk chemical (butyric acid).

Keywords Butyric acid · *Clostridium tyrobutyricum* · Biomass resource · Fibrous-bed bioreactor · Immobilization

Introduction

The exploitation of cheap, renewable biomass as alternative sources for valuable fuels and products, which leads to a new manufacturing concept, generally referred to as the biorefinery, has been strongly stimulated by the rapidly increasing concerns about the future

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scarcity, cost, and environmental impact of fossil fuel. In future biorefineries, biofuels and chemicals will be produced from biomass resources, including corn grains and lignocellulosic biomass (such as agricultural residues, forestry wastes and thinnings, waste paper, and energy crops). A recent report [1] has suggested that in the near term, more than 1.3 billion tons of biomass could be produced annually in the United States on a sustainable basis, mostly from agricultural and forestry sources. Another study [2] has shown that biomass has the potential to simultaneously meet the nation's needs for liquid transportation fuel and for food, feed, and fiber, provided that we develop more advanced technologies and make certain land-use changes that would not require more net land. The cost-competitive production of biofuels and chemicals is currently prevented by the high cost of biomass feedstocks and the processes for converting biomass to sugars—that is, the cost of the thermochemical pretreatment and enzyme hydrolysis unit operations in a biorefinery. At present, many chemical and biochemical processes have been developed to utilize a variety of plant biomass to produce glucose and xylose, the two major constituents of sugars, which can be further fermented by microorganisms to produce ethanol and butanol as the alternative for the oil-based fuel [3, 4].

Butyric acid is one of the short-chain fatty acids with many applications in chemical, foodstuff, and pharmaceutical industries. It can be used as the pure acid to enhance butter-like notes in food flavors or in the form of esters as additives for increasing fruit fragrance, and as aromatic compounds for the production of perfumes [5]. The role of this acid in the treatment of hemoglobinopathies, cancer, and gastrointestinal diseases is also well-known [6–10]. Currently, butyric acid is synthesized commercially via petrochemical routes. However, the demand for butyric acid from microbial fermentation is high due to a strong preference by consumers and manufacturers for using bio-based natural ingredients in foods, cosmetics, and pharmaceuticals. It is thus of increasing interest to produce butyric acid from the fermentation of biomass resources. There are several bacterial strains that produce butyric acid. Production is an anaerobic process and the producers are strict anaerobes. Production strains belong to the genera *Clostridium*, *Butyrivibrio*, *Butyribacterium*, *Sarcina*, *Eubacterium*, *Fusobacterium*, and *Megasphera*. The species *Bacteroides melaninogenicus*, *Treponema phagedenis*, and *Peptococcus asacelarolyticus* are also known as butyrate producers [11, 12]). Among them, *Clostridium* species have been used preferably for butyric acid production due to their simple medium requirement for cell growth and relatively high product yield [13, 14]. Recently, one effective two-stage bioprocess is developed to produce butanol from glucose [15]. This process includes butanol production from butyrate with immobilized cells of *Clostridium tyrobutyricum* and *Clostridium acetobutylicum*, which doubled the overall butanol yield from glucose compared with the traditional acetone-butanol-ethanol fermentation. Butyrate and hydrogen can also be produced effectively from xylose with *C. tyrobutyricum* [16]. Therefore, it is very important to optimize this fermentation process for cost-effective production of butyrate with *C. tyrobutyricum*.

Compared with the traditional suspended-cell fermentation, immobilization of microbial cells has some attractive characteristics such as increasing the biomass in the reactor and the productivity of fermentation, preventing the loss of cells, facilitating continuous fermentation, etc [17]. Recently, fibrous materials have been developed for cell immobilization because of their high specific surface area, high void volume, low cost, high mechanical strength, high permeability, and low press drop. A fibrous-bed bioreactor (FBB) with cells immobilized in the fibrous matrix packed in the reactor has been successfully used to produce several organic acids (lactic acid, acetic acid, and propionic acid) with significantly improved reactor productivity, final product yield and concentration

[18]. The advantages of the FBB include efficient and continuous operation without repeated inoculations, elimination of cell lag phase, good long-term stability, and easier downstream processing. These advantages could be attributed to the high viable cell density maintained in the bioreactor. In the present work, the FBB system was employed to immobilize the cells of *C. tyrobutyricum*. Butyric acid production between free and immobilized cell fermentation was compared. The results showed that immobilized cells in FBB were very effective to improve the performance and economics of butyric acid fermentation process, and xylose could also be well utilized to produce butyric acid with high yield and final product concentration.

Materials and Methods

Microorganism and Maintenance

Clostridium tyrobutyricum ATCC 25755 was supplied by professor Shang-Tian Yang from Ohio State University and preserved at 4 °C in anaerobic conditions on *Clostridium* growth medium [19].

Culture Media and Cultivation Condition

The fermentation medium contained per liter of distilled water: 30 g glucose (or 25 g xylose), 5 g yeast extract, 5 g peptone, 3 g$(NH_4)_2SO_4$, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.03 g $FeSO_4 \cdot 7H_2O$. The cultivation conditions are 37 °C, pH 6.0, and anaerobic fermentation with N_2.

Fibrous-Bed Bioreactor

The bioreactor system consisted of a 5-L stirred-tank fermentor (B. Braun, B. Braun Biotech International, Germany), which was connected to a packed glass column reactor (∅50 mm×310 mm), with a water jacket through a recirculation loop (Fig. 1a). A piece of cotton towel (185×300 mm; ~2 mm in thickness; with >95% porosity) laid with a stainless steel mesh was spiralled and placed inside the column for cell immobilization (Fig. 1b). A 20- to 30-mm layer of glass spring was filled in the bottom of column to support the spirally wound matrix and create a homologous flow distribution. The working volume of the glass column reactor was 500 mL.

Bioreactor Setup and Operation

Before use, the bioreactor was autoclaved for 30 min at 121 °C, held overnight, and then autoclaved again for another 30 min for complete sterilization. The entire reactor system contained ~3 L of the medium. Anaerobiosis was maintained by sparging the medium with N_2. The reactor temperature was kept at 37 °C, agitated at 150 rpm, and pH controlled at 6.0 for maximum productivity and yield [20] by adding 6 N NaOH facilitated by an in-line sensing and dosing system. At the beginning of fermentation, ~150 mL of cell suspension in a serum bottle was inoculated into the fermentor and allowed to grow for 3 days until the cell concentration reached ~4.0 g L^{-1}. Cell immobilization was then carried out by circulating the fermentation broth through the fibrous bed. After about 2 days of continuous circulation, the fermentation broth in the fermentor was replaced with fresh medium to start

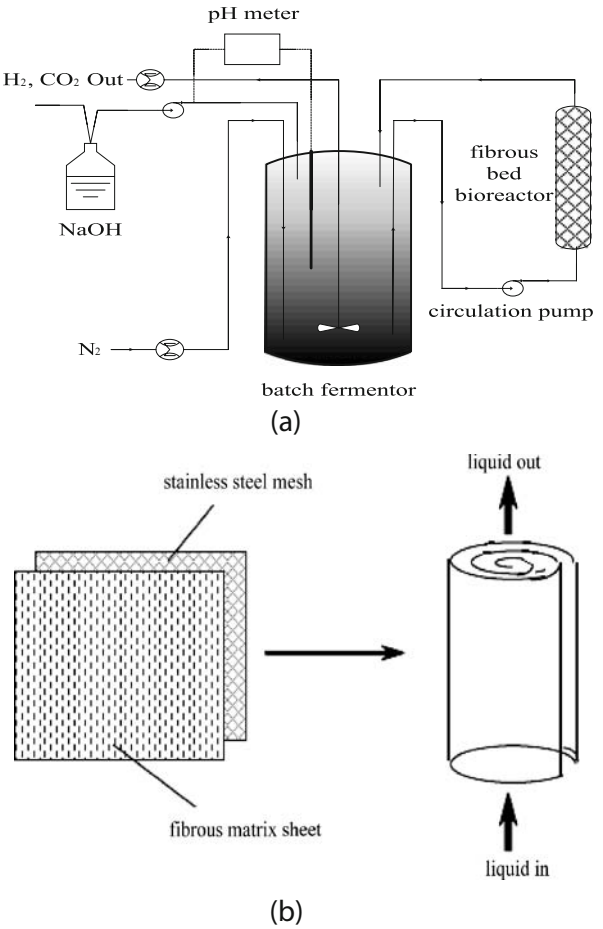

Fig. 1 The immobilized FBB system used for the butyric acid fermentation. (a) Reactor system, (b) the construction of the fibrous-bed bioreactor

a new batch. The reactor was then operated at the fed-batch mode by pulse feeding concentrated sugar (900 g L^{-1} glucose or 750 g L^{-1} xylose) when the sugar concentration in the fermentation broth decreased to zero. To evaluate the maximum butyric acid concentration achievable in the fermentation, the feeding was continued until the fermentation ceased to produce butyrate due to product repression.

Analytical Methods

Cell density was analyzed by measuring the optical density of the cell suspension at a wavelength of 600 nm (OD_{600}) with a spectrophotometer (Ultrospec 3300 pro, Amersham Bioscience). One unit of OD_{600} corresponded to 0.68 g L^{-1} cell dry weight for cells growing in the glucose medium and 0.797 g L^{-1} in the xylose medium [16]. The glucose and xylose concentrations were measured using Sugar Content Measuring Device (SBA-40C) and DNS method, respectively [21]. Quantitative analysis of butyric acid and acetic acid was performed by gas chromatography (Agilent 6820 GE, Agilent Technologies) [22].

Results and Discussion

Production of Butyric Acid from Glucose with Suspended-cell Fermentation

The kinetics of butyric acid fermentation of glucose with *C. tyrobutyricum* is shown in Fig. 2. Biomass production was minimal for the first 20 h, after which there was a period of relatively rapid growth that reached stationary phase in the middle of the second batch. Production of butyric acid and acetic acid was low after inoculation but increased afterward and reached the peak values (7.05 g L^{-1} butyric acid, 2.11 g L^{-1} acetic acid) in the first batch. Then, concentrated glucose solution was supplemented into the system at 38 h for continuous production of butyric acid. After another 40 h of fermentation, the concentration of butyric acid and acetic acid reached 13.40 g L^{-1} and 3.69 g L^{-1}, respectively. The total yield of butyric acid in this glucose-fed fermentation was 0.24 g g^{-1} (glucose), and glucose feeding did not improve the yield but significantly improved the volumetric productivity.

Production of Butyric Acid from Glucose in the Immobilized-cell FBB System

For the purpose of comparison, repeated runs of batch fermentation in the FBB were carried out at the same culture conditions as the suspended-cell fermentation. Figure 3 shows the fermentation kinetics of butyric acid production from glucose with immobilized cells of *C. tyrobutyricum*. As shown in Fig. 3, the immobilized-cell fermentation not only was faster but also produced a much higher butyrate concentration than those obtained in suspended-cell fermentation. The highest butyric acid concentration produced in three repeated batches increased 95%, 100%, and 90% (13.73, 14.14 and 13.43 g L^{-1} vs. 7.05 g L^{-1}), respectively, and the product yield was in the range of 0.45–0.47 g g^{-1} (vs. 0.24 g g^{-1}). The acetic acid production was not affected as obviously as the butyric acid production in the FBB system with regard to its synthesis in the suspended-cell fermentation (3.11 g L^{-1} vs. 2.11 g L^{-1}). Apparently, in the FBB system it is very easy to carry out the repeated batch fermentations with higher productivity. The similar kinetics of glucose consumption, product production, and byproduct formation suggested that this system was very stable and easily scaled up.

Fed-batch fermentation in the FBB system was performed to evaluate the possibility of increasing the volumetric productivity of butyric acid by glucose feeding. The concentration of butyric acid decreased transiently then continued to increase following the pulsed addition of glucose (Fig. 4). The final concentration of butyric acid reached 24.88 g L^{-1} after three batches, yet the yield was lower than the previous repeated fermentation (0.31 g g^{-1} vs. 0.47 g g^{-1}). The butyric acid yield from glucose varied

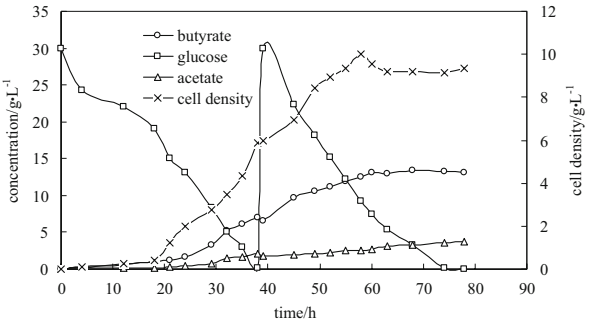

Fig. 2 Kinetics of butyric acid fermentation from glucose with free cells of *Clostridium tyrobutyricum* at 37 °C and pH 6.0

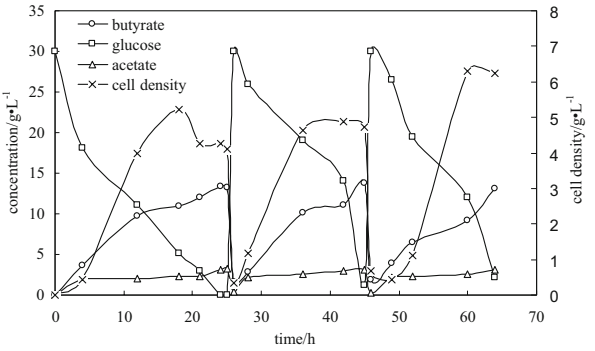

Fig. 3 Kinetics of repeated-batch fermentation of butyric acid from glucose with immobilized cells of *Clostridium tyrobutyricum* in the FBB at 37 °C and pH 6.0

between 0.28 and 0.44 g g^{-1} in the immobilized-cell fermentation, with a lower yield (0.28 g g^{-1}) in the third batch when more butyrate accumulated. The concentration of acetic acid increased fast at the beginning of the fermentation, which can be readily interpreted since acetic acid production yields more ATP than butyric acid production and is, as a consequence, better suited to meet the energy demands of rapid growth. However, the production of acetic acid stopped when cell growth was inhibited by a high concentration of butyric acid and the final concentration of acetic acid increased only by 45% (4.50 g L^{-1} vs. 3.10 g L^{-1}), which will be particularly beneficial for downstream separation of butyric acid from the broth. We also found that the cell density in the broth was lower than that in the suspended-cell fermentation (6.30 g L^{-1} vs. 10.00 g L^{-1}) (Fig. 4). It is possible that there might be a high density of cells immobilized in the fibrous matrix, which contributed to the higher fermentation rate, but the immobilized cells probably did not grow as much as the free cells suspended in the medium.

Production of Butyric Acid from Xylose with Suspended-cell Fermentation

Xylose and glucose are the two most abundant sugars in biomass resources. However, bioconversion of xylose to chemicals has been limited by the preference of microorganisms for glucose as carbon source and energy source. Glucose-mediated catabolic repression may be one of the factors. However, inhibition of xylose uptake was not observed in butyrate fermentation by *C. tyrobutyricum* [23]. As shown in Fig. 5, negligible amounts of butyric acid and acetic acid were produced in the first 30 h after inoculation. Then butyric acid and

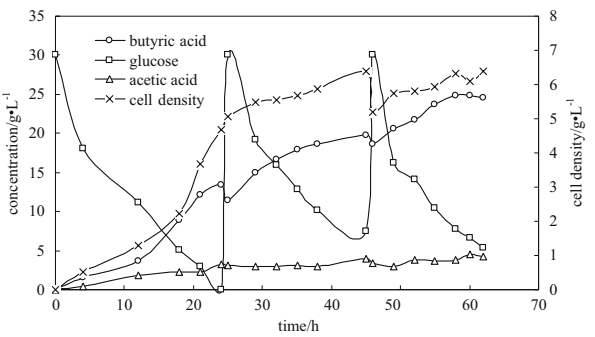

Fig. 4 Kinetics of fed-batch fermentation of butyric acid from glucose with immobilized cells of *Clostridium tyrobutyricum* in the FBB at 37 °C and pH 6.0

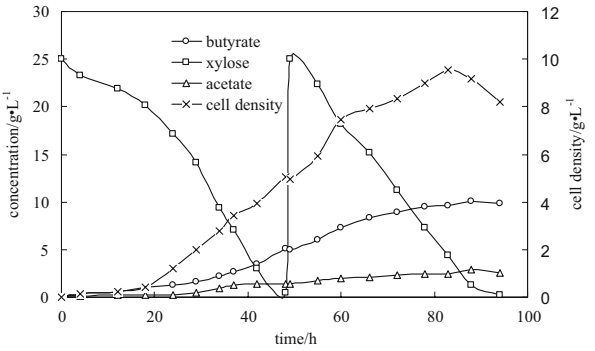

Fig. 5 Kinetics of butyric acid fermentation from xylose with free cells of *C. tyrobutyricum* at 37 °C and pH 6.0

acetic acid were simultaneously produced with a fast increase in concentration, which reached the peak values (5.05 and 1.38 g L^{-1}, respectively) at the end of the first batch. With concentrated 25 g L^{-1} xylose supplemented into the system, the concentration of butyric acid and acetic acid continuously increased to 10.03 and 2.85 g L^{-1}, respectively, which was lower than the fermentation with glucose, the same as the specific growth rate and biomass yield (see Table 1). The different growth rates on different sugars are probably due to the lower energy efficiency of growth on xylose compared with glucose. The metabolic pathway from glucose to pyruvate is different from the pathway from xylose to pyruvate.

$$\text{Glucose} + 2\text{NAD} + +2\text{ADP} \quad 2\text{Pyruvate} + 2\text{NADH2} + 2\text{ATP}$$
$$3\text{Xylose} + 5\text{NAD} + 5\text{ADP} \quad 5\text{Pyruvate} + 5\text{NADH2} + 5\text{ATP}$$

Following transport into the cytoplasma, glucose is metabolized to pyruvate via the Embden–Meyerhof–Parnas (EMP) pathway. Pentoses, such as xylose, are metabolized by pentose–phosphate pathway. It has been reported that extra energy consumption is required for the transport of xylose across the cell membrane [24], which is usually energized

Table 1 Comparison of cell growth and acid production with different sugar sources between free-cell and immobilized fermentations.

	Free cell		Immobilized cell	
Sugar Sources	Glucose	Xylose	Glucose	Xylose
Cell Growth				
Special growth rate (h^{-1})	0.16±0.04	0.07±0.10	0.17±0.008	0.14±0.010
Biomass yield (g g^{-1})	0.15±0.018	0.18±0.021	0.16±0.010	0.20±0.011
Acid Production				
Butyric acid conc. (g L^{-1})	7.05±0.23	5.05±0.26	13.73±0.31	10.05±0.39
Butyric acid yield (g g^{-1})	0.24±0.030	0.20±0.028	0.46±0.020	0.40±0.020
Acetic acid conc. (g L^{-1})	2.11±0.45	1.38±0.50	3.10±0.10	2.08±0.080
Acetic acid yield (g g^{-1})	0.06±0.010	0.05±0.013	0.10±0.012	0.08±0.011
B/A ratio	3.34±0.50	3.66±0.52	4.43±0.49	4.83±0.58

The data with immobilized cells were obtained in the repeated batch fermentation in the FBB.

B/A ratio means the ratio of butyric acid to acetic acid based on yield.

directly by a high-energy phosphate compound, instead of a phosphoenolpyruvate (PEP)-dependent phosphotransferase system commonly used for glucose transport. Therefore, the net ATP yield from xylose was lower than that from glucose.

Production of Butyric Acid from Xylose in the Immobilized FBB System

For economical production of butyrate from xylose-containing biomass resources, it is critical to have a high butyrate yield, concentration, and reactor productivity in the fermentation. The objective of this study was to evaluate possible effects of xylose uptake and conversion to butyric acid in the FBB fermentation system. As shown in Fig. 6, the kinetics of butyric acid production from xylose is similar with that from glucose in these immobilized systems. The highest product concentration in the three repeated batches was around 10 g L^{-1} when 25 g L^{-1} xylose was introduced to the immobilized bioreactor. Although glucose seemed to be more suitable for butyric acid production, the product yield with xylose as the substrate was also very high (0.40 g g^{-1}). Furthermore, the selectivity of butyric acid production (butyrate/ acetate ratio) in these xylose-based repeated batches reached up to 4.83, a little higher than that (4.43) in glucose-based repeated batch fermentations. Considering the importance of utilizing xylose in the biorefinery, the above results would stimulate further studies to develop new strategies for converting xylose to biofuel and some value-added chemicals.

Conclusion

In the present work, the FBB fermentation produced a high concentration of butyric acid of 24.88 g L^{-1}, which is higher than that from the suspended-cell fermentation (13.70 g L^{-1}). Meanwhile, as shown in Table 1, the product yield in FBB was much higher than that from suspended-cell fermentation (0.46 g g^{-1} vs. 0.24 g·g^{-1} with glucose, and 0.40 g g^{-1} vs. 0.20 g g^{-1} with xylose). The results also clearly show that fed-batch fermentation with immobilized-cell allowed good selectivity of butyric acid (butyrate/acetate ratio 4.43:3.34 with glucose, and 4.83:3.66 with xylose). The increased butyrate yield in the FBB fermentations can be partially attributed to reduced cell growth. Therefore, less acetic acid was produced as reduced cell growth needs less energy and more substrate could be

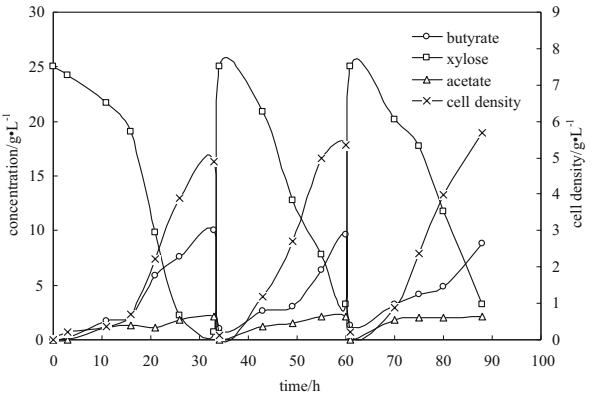

Fig. 6 Kinetics of repeated-batch fermentation of butyric acid from xylose with immobilized cells of *C. tyrobutyricum* in the FBB at 37 °C and pH 6.0

metabolized to butyric acid. It has been reported that culturing in the FBB facilitates the adaptation and selection of mutants because of the high viable cell densities (more than 50 g L^{-1} cell dry weight in the fibrous bed volume) maintained in the conducive environment provided by the fibrous matrix. Thus, the effect of cell adaptation in the FBB partially leads to the improvement of the product yield and final concentration. In addition, the fed-batch strategy will improve the final butyrate concentration greatly, indicating the high potential of improving the productivity of butyrate in the fibrous-bed bioreactor.

Similar fermentation results (kinetics and final product yield) were achieved in the immobilized FBB systems when xylose was fermented by *C. tyrobutyricum*. Compared to glucose, xylose fermentation would lead to a lower specific growth rate. This might be explained by lower energy efficiency in *C. tyrobutyricum* with xylose as the sole carbon source. With high product yield (0.40 g g^{-1}) and higher selectivity (butyrate/acetic acid ratio 4.83), xylose can be efficiently used in butyric acid fermentation, which has no obvious preference for either glucose or xylose. The investigations of immobilized fermentations in FBB and xylose as the substrate for butyrate production showed the potentials of butyrate fermentation as a very promising strategy of biorefinery. Further, studies will be necessary for the commercial production of butyric acid from renewable biomass resources.

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