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ARTICLE TYPE

Sweet sorghum bagasse as an immobilized carrier for ABE fermentation by using *Clostridium acetobutylicum* ABE 1201 †

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In this study, sweet sorghum bagasse was used as immobilized carrier for ABE fermentation. The SEM reveals the relationship between *Clostridium* cells and sorghum bagasse in the shape of adsorption and embedding. The ABE productivity and the yield of ABE solvents in batch fermentation were 0.37 g/ L·h and 0.41 g/g, with 68 % and 24 % higher than free cells fermentation, respectively. Under the optimized condition, repeated fed-batch fermentation was operated. Furthermore, a total of 970 h of continuous fermentation at different dilution rates was performed. The maximum ABE concentration of 16.5 g/L was obtained at a dilution rate of 0.08 /h with balanced superiority in both ABE production and productivity. This novel immobilization method using sweet sorghum bagasse showed attractive prospect for industrial production of biobased butanol.

I. Introduction

Due to the depletion of fossil fuel resources and the increasing price of crude oil, alternative resources has been paying closer attentions to during past decades [1]. Since it owns the properties of higher energy density, higher air-fuel ratio, lower research octane number and motor octane number [2], biobutanol, the production via acetone-butanol-ethanol (ABE) fermentation, has been recognized as a superior biofuel to ethanol [3]. However, limited by end product toxicity, traditional batch ABE fermentation based on free cell culture suffered from low cell density, low reactor productivity as well as relative high down times, which result in the competitive weakness in compared with the petrochemical based butanol [4-6]. Another weakness of ABE fermentation is that the strains will gradually lose the ability to produce solvents during repeated inoculated or continuous cultivation [7, 8]. It was also found that concentrated microbial cells are of great important to achieve high volumetric productivity [9].

In order to achieve high cell density in ABE fermentation, the technique called cell immobilization has been wildly applied in it. This technique can help adhering bacteria on the surface of immobilized carrier by electrostatic adsorption or embedding bacteria on the caves of immobilized carrier surface structure, which absolutely has superiority of maintaining a highly viable cell density in the bioreactor [10]. It is also an ideal way to eliminate downtime of fermentation and keep the strains stable at high productivity [9]. The previous study suggested that a variety of support matrices for immobilization not only had potential possibilities for higher ABE production, but also showed good properties of integrating solvent separation [11, 12]. Among these immobilized carriers, a variety of natural lignocellulosic materials such as wood pulp, corn stalk and sugarcane bagasse, have been treated as natural supports [1, 13, 14]. However, many of these supports show a few drawbacks due to its non-uniform structures, which lead to the little facilitating of fermentation because of the poor immobilization behaviors of cells [15]. More importantly, it was difficult to find out an ideal immobilization carrier to achieve the objective of both high ABE concentrations and high productivity concurrently, which would result in a lower purification cost and enhancing the process efficiency at the same time.

Sweet sorghum, providing more raw fermentable sugars under marginal conditions than other sugar crops, is considered to be a competitive industrial crop under biorefinery concept [16, 17], and attracted more and more scholar and decision makers's attention recently [18]. According to the previous studies, the biological composition and structure of sweet sorghum fiber is different sharply from other relative crops, such as corn stalk and sugarcane [19-21]. Therefore, compared with other lignocellulosic materials, sweet sorghum bagasse might be treated as excellent immobilization carrier because of its unique performance and superiorities. Our previous study suggested sweet sorghum bagasse is a good immobilization carrier for ethanol production (the fungal fermentation system) [15, 22]. Nevertheless, as far as the authors can know, no studies have been performed to use sweet sorghum bagasse as bacteria immobilized carrier for ABE fermentation up till now because as immobilized carrier, the bacterial fermentation system definitely has so many different characteristics when compared with fungi's.

The objective of this work is to evaluate the applicability and performance of sweet sorghum bagasse as carrier for

immobilization of *Clostridia*. Sizes and the loading of carrier for ABE fermentation were optimized. The performance of repeated fed-batch immobilized fermentation was studied. Additionally, the effectiveness of continuous one-stage immobilized ABE fermentation with different dilution rate was also investigated.

II. Experimental section

A. Chemicals and Materials

Sweet sorghum was cultivated on experimental plot by Chinese Academy of Agricultural Sciences at Shunyi District, Beijing. After squeezing out the fermentable juice, the skin and the outside fiber were removed. Then the bagasse was soaked in water until the residue sweet juice was washed out, which is to eliminate interruptions of fermentable sugars. The chopped stalk was then dried, with approximately 70 mL moisture vaporized from every 100 g stalk. The dried bagasse was then sieved to remove fine and larger particles.

B. Strain and culture conditions

C.acetobutylicum strain ABE 1201 derived from ATCC 824 was used in this study [23]. The culture for ABE fermentation was prepared as described in our previous study, containing 60 g/L glucose, phosphate buffer containing 1 g/L KH_2PO_4 / K_2HPO_4, 1 mg/L para-amino-benzoic acid and 0.01 mg/L biotin as vitamins, in addition to these, 2.2 g/L ammonium acetate and mineral for bacteria grow were added (0.2 g/L $MgSO_4{\cdot}7H_2O$, 10 mg/L $MnSO_4{\cdot}H_2O$, 10 mg/L $FeSO_4{\cdot}7H_2O$). After loading the carriers into the medium, acetic ammonium and vitamin were sterilized by filtering separately, and oxygen free N_2 was swept across the surface of the medium for 15 min to maintain an anaerobic environment. After 25 min sterilization at 116 ^oC, the fermentation inoculated with 10 % of active cells started at 37 ^oC without stirring.

C. Batch fermentation

Batch fermentation was carried out at 37 ^oC in 1 L glass bioreactor with 600 ml fermentation volume. The bioreactor contained sweet sorghum bagasse with different sizes and different ratio of liquid to carrier material. Then the medium was inoculated with 10 % highly motile cells of *C. acetobutylicum* ABE 1201. During fermentation, samples were taken at constant time interval to analyze concentration of acetone, butanol, ethanol and residual sugar. The yield was calculated from the ratio of the total solvents produced to the total glucose utilized [40].

All the batch operations were carried out in duplication and the reported results are the average of two fermentations.

D. Repeated fed-batch fermentation

As for repeated batch fermentation, according to the method of Yu et al. [15], after 60 h of batch fermentation, most of fermentation broth in the 1 L glass bioreactor with 600 ml working volume was pumped out. The immobilized carrier with hyper strains was remained in the fermentor as seed. Then, pumped fresh medium into the fermentor and took batch fermentation in this way one by one. The immobilized cells system was repeated for 10 feeding cycles over 610 h. In the process of fermentation, samples were taken at constant time interval to analyze concentration of acetone, butanol, ethanol and residual sugar [40].

E. Continuous fermentation

Operation of the continuous fermentation was performed according to the previous study with a little modification [1]. Single-stage continuous immobilized fermentation was carried out. The bioreactor contained sweet sorghum bagasse (size, 1-2 cm) with the 1:20 (w/v) ratio of carrier material to liquid. The fermentation was allowed to proceed in batch mode for 48 h, then the sterilized medium was continuously pumped into the bioreactor at different dilution rates. The working volume of the fermentor was kept in constant as feed rate, by means of removing excess medium from the bioreactor. Fresh medium was continuously fed to the fermentor using a peristaltic pump (Longer Precision Pump Co., Ltd., Baoding, China). Dilution rates varied from 0.08-0.32 /h, with reactor temperature of 37 ^oC. The steady state was confirmed by stable product values at a specific dilution rate. When the system reached the steady state, solvent productivity was calculated as the total solvents (sum of ABE) or butanol produced multiplied by the dilution rate. At this point, the dilution rate is defined as the ratio between feed flow rate and reactor volume (600 ml) [40].

F. Analytical methods

A high performance liquid chromatography (HPLC) equipped with a refractive index detector (RID, Shimadzu LC-10A, Japan) was used to analyze the concentration of residue glucose contained in the fermentation broth. An Aminex HPX-87P carbohydrate analysis column (Bio-Rad Labs, USA) was operated at 65^oC. Deionized water was used as the mobile phase with 0.6 ml/min of flow rate.

The fermentation products, including acetone, butanol, ethanol, acetic acid and butyric acid were detected by a gas chromatograph (GC, Shimadzu GC-2010, Japan), which was equipped with a flame ionization detector（FID） and a 2 m long glass column packed with Porapack Q 80/100 mesh. N_2 was used as the carrier gas here. Both injector and detector temperatures were 230 ^oC, and the column temperature was 120 ^oC for 0.5 min, increasing 20 ^oC /min to 180 ^oC, and holding for 3 min at 180 ^oC, increasing 30 ^oC /min to 230 ^oC, holding for 10 min at 230 ^oC. External standard method was used to determine the concentration of solvents.

G. Analysis of the carrier strauctur e

The structure of carrier was studied with scanning electron microscope (SEM). Pretreatment of immobilized carrier was routinely obeying the method of previous study. The carriers were immersed in 3.5 % glutaraldehyde for 6 h, and then dried with 50 %, 70 %, 90 %, 95 % and 100 % of ethanol respectively,

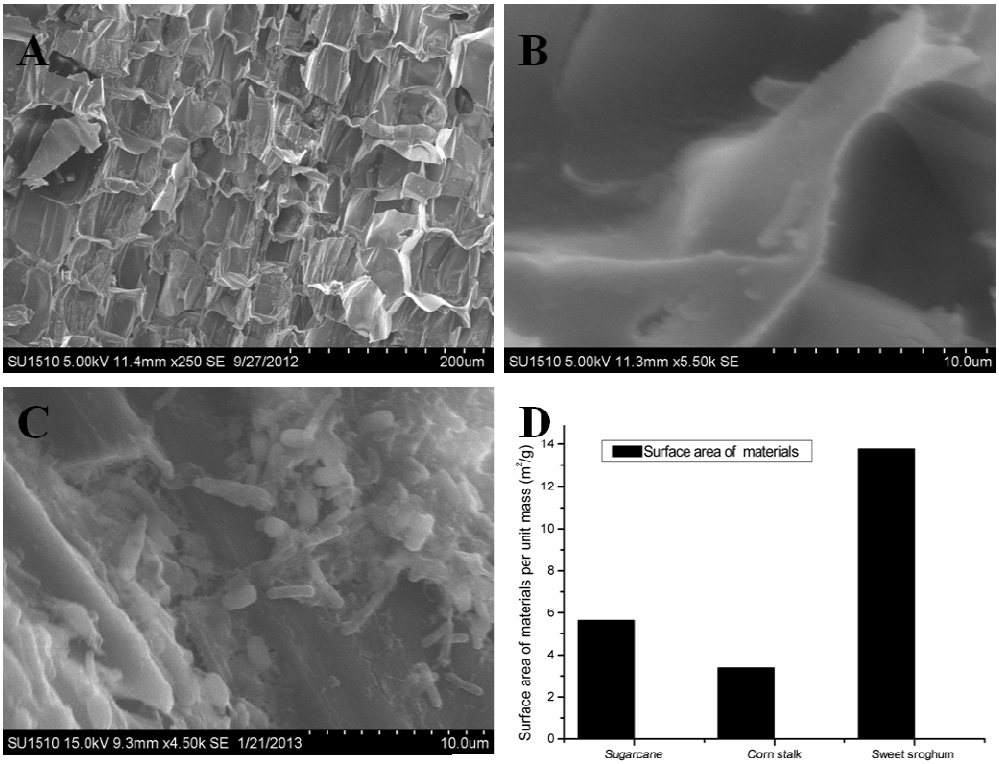

Fig.1. Scanning electron microscopic images of immobilization carriers. (A) alveolate structure of the carrier without loading *C. acetobutylicum* ; (B) Structure inner the alveolate, it showed a more glabrous surface of the bagasse with several pores and wrinkles; (C) *C. acetobutylicum* cells adhered onto the rough surface of sweet sorghum bagasse, cells were immobilized in the cavums of the stalk cells firmly, the wrinkles structure of the bagasse was provide a large area for cell adherence, while the alveolate structure could also provide a stable microenvironment to the strains' metabolism; (D) The specific surface area per unit mass of sugarcane bagasse, corn stalk bagasse and sweet sorghum bagasse. The structure of sweet sorghum bagasse was matchless with its large surface area, which could more or less contribute to the metabolism of strains.

followed by overnight retention in a desiccator to remove moisture. The samples were scanned and photographed with a scanning electron microscope (Hitachi Su1510, Japan). The specific surface area was obtained by the standard BET method apparatus (V-Sorb 2800S Series, Jinaikang Co., Ltd, Wuhan, China).

III. Results and discussion

A. Analysis of immobilization carrier

Lignocellulosic materials, although act as supports in almost all of the immobilization methods, have the limitation of operational stability because of poor cell desorption [15]. The immobilization efficiency and cell absorption on to sweet sorghum bagasse were examined using scanning electron microscope (Fig.1.). In general, compared with the clean surface of the non- inoculated alveolate alveolar structure showed in Fig.1A, the sweet sorghum bagasse showed affable cell adherence due to its alveolar structure in Fig.1B. Since the roughness of carrier was recognized as the main factor of immobilized systems ability [24], the surface structure of sweet sorghum bagasse provided an ideal large specific surface area so that the cells could easy attach to the surface of the bagasse and grow on the porous surface. In this way, it greatly increased the contact surface of the substrate to microorganisms and improved the cell culture density [25]. Additionally, the porous surface of sweet sorghum bagasse constructed a favorable extracellular microenvironment to promote cell proliferation and metabolism [26].

Since the specific surface area of the bagasse alveolate structure could affect the adherence of stains to the immobilized carrier and the inner microenvironment for cells metabolism, respectively, the specific surface area of different types of sweet stems was further invested. As it was illustrated in Fig. 1D, compared with other two difference bagasse, sweet sorghum bagasse provided a considerably large specific surface area per unit mass (13.78 m^2/g). Therefore, the sweet sorghum bagasse was the vivid one with large specific surface area. When used as immobilized carrier in ABE fermentation process, the large specific surface area would increase the probability of strains adherence, which contribute to the stable of microenvironment of cells metabolism.

B. Comparison of batch fermentation in free cell system and immobilized cell system

Immobilized cell technology was considered to have many advantages over conventional systems, and displayed an

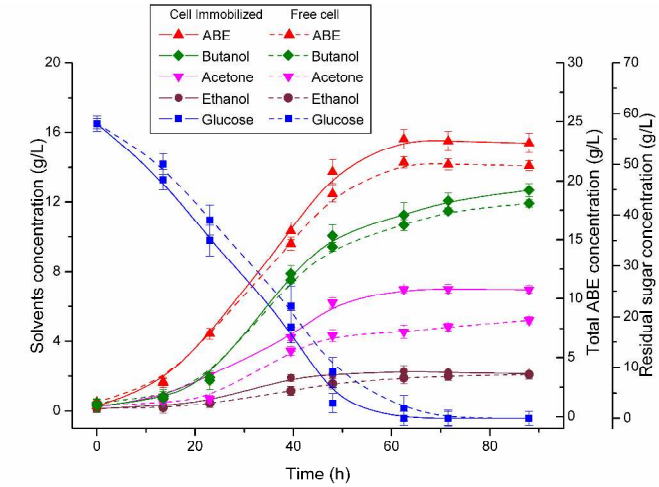

Fig. 2. The comparison of the fermentation kinetics between immobilized cells system and free cells system with the initial sugar concentration of 60 g/L.

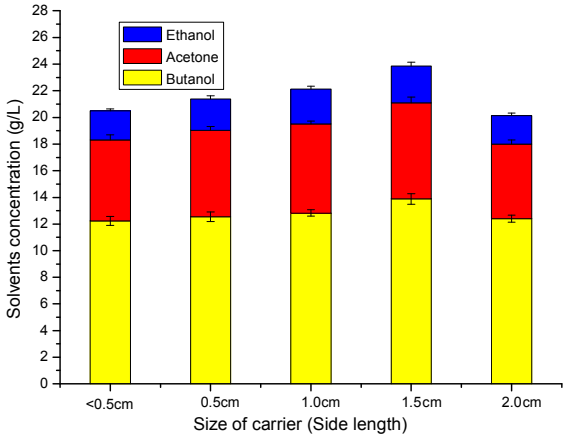

Fig.3. Effect of sorghum bagasse sizes on immobilized cell system

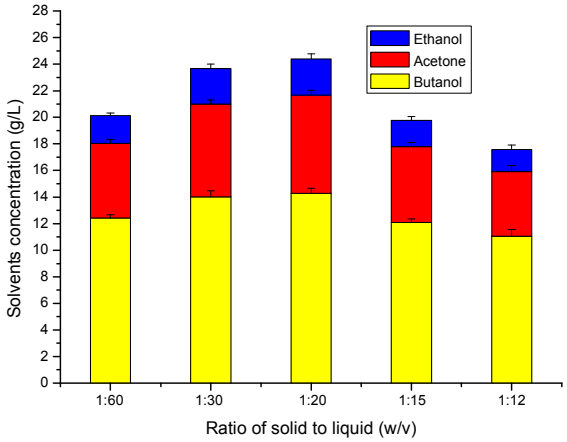

Fig.4. Effect of solid loading on immobilized cell system

increasing products productivity compared to free cells [27, 28]. On the other hand, however, some immobilized systems got lower productivity. The reason that the physiology of immobilized cells differed from that of free cells was caused by several factors [29], including nutrient limitations and microenvironments surrounding the cells. These factors were recognized as the key of the change of cell physiological and morphological after immobilization [30]. Thus, parameters of immobilized fermentation might be sharply different with the free ones.

As is shown in Fig. 2, experiments were carried out with the same initial sugar concentration of 60 g/L to make comparison of batch fermentation between in free cell system and in immobilized cell system. In general, almost all of the initial sugar in both two systems was utilized, with 0.33 g ABE/g consumed sugar in the free cell system and 0.41 g ABE/g consumed sugar in the immobilized cell system. In the free cells system, after 88 h of fermentation, a total of 19.21±0.26 g/L ABE (2.08±0.16 g/L of ethanol, 5.21±0.21 g/L of acetone and 11.92±0.22 g/L of butanol) was yield, the productivity of ABE was ~0.22 g/L·h. In sharp contrast, the fermentation period was ended after 63 h in the immobilized cells system with an ABE yield of 23.26±0.18 g/L (2.27±0.32 g/L of ethanol, 6.97±0.21 g/L of acetone and 14.02±0.29 g/L of butanol), while the productivity of ABE in the immobilized cells system was ~0.37 g/L·h. Therefore, the fermentation period of the immobilized cell system was almost 28.4 % shorter than that of the free cells system, which is similar with the previous studies in using different types of immobilized carriers [31-33]. As for ABE productivity, with the shorter fermentation period and higher ABE concentration, the productivity of ABE solvents in the immobilized cell system got 1.68 times higher than that of the free cells system.

In fact, the cost of biobutanol would be similar to that of synthetic butanol when volumetric productivity of biobutanol was increased by about 50 % [34]. Woods (1995) also stated the ABE fermentation should be possible for industrial production if the final solvent concentration could be increased by one-third (i.e. to the levels of 22-28 g/L), and the batch fermentation time of could be kept between 40 to 60 h [35]. Similarly, when comparing with these forecasts, it could be found that the present process of the immobilized ABE fermentation with sweet sorghum bagasse as immobilized carriers can basically meet the former predict of industrially viable.

C. Effect of sizes and solid loading on immobilized cell system

Most of the immobilized cells are embedded in the cavum of the stalk cells [36]. With the same total masses, bigger sorghum bagasse has more intact stalk cells than the small one. As a result, more cells could be immobilized in each unit. However, with the size of carrier increasing, the mass transfer in the inner of the sorghum baggase will become more difficult because of the increasing inner mass transfer resistance, which will finally influence the fermentation productivity [15]. As is shown in Fig.3, the effect of immobilized carrier was obviously. The best size of the sorghum bagasse was 1.5 cm ×1.5 cm ×1.5 cm. Under this condition, about 13.88± 0.38 g/L of butanol and 23.87± 0.31 g/L of total ABE were yield after 60 h of fermentation.

Solid loading is another key of immobilized cell system [37]. For batch fermentation system, solid loading would effect on the heterogeneous of immobilized cell populations and mixing of the multiphase immobilized cell reactor. Fig.4. shows the influence

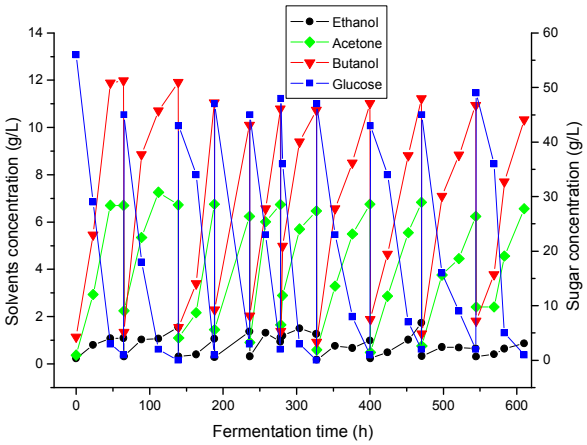

Fig.5. Results of repeated batch fermentation by sweet sorghum bagasse with immobilized cells

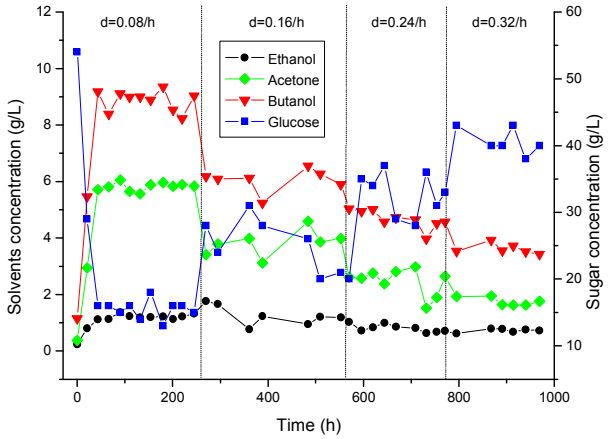

Fig.6. Continuous ABE fermentation with different dilution rate in using sweet sorghum bagasse as immobilizated carrier

of solid loading on immobilized cell system. The result indicated that the optimal solid loading of immobilized ABE fermentation was 1:20 (w/v), about 14.28± 0.35 g/L of butanol, 7.39± 0.36 g/L of acetone and 2.73± 0.37 g/L of ethanol (total ABE was 24.4± 0.28 g/L) were produced from 60 g/L glucose consumed in 60 h. Once the solid loading fluctuated, a correlation of lower productivity and solvents were yield. It might be attributed to small variations in loading and specific gravity which were reported to have significant influences on air flow rate and mixing time of immobilized system [38].

D. Repeated fed-batch fermentation of immobilized cells system

Repeated fed-batch fermentation was further operated with the optimized conditions to evaluate the long-term stability of the immobilized cells system (Fig.5). In general, the product profiles in the fermentation broth were similar to those of the previous batch experiment. As expected, repeated batch fermentation with 44-48 g/L initial glucose of each batch produced 10-12 g/L butanol, 6.5-7.5 g/L acetone and 1-1.5 g/L ethanol (total ABE: 18-21 g/L). The overall product yields from glucose consumed were (g/g): butanol 0.23-0.25, acetone 0.15-0.16, ethanol 0.02-0.03 (total ABE: 0.41-0.44), which were similar to batch immobilized fermentation.

Clearly, repeated fed-batch immobilized fermentation of butanol with sweet sorghum bagasse as carrier had long-terms stability. It might be caused by the fact that immobilized cells contained much higher percentage of saturated fatty acids compared with free cells, which led to greater product tolerance in the immobilized cells. Hence, greater survival and cell activity in subsequent cycles compared to free cells can be observed [39].Another theory of the long-term fermentation activity is that the immobilized cells might retain enzyme activity for a long time due to the different compositions of cells compared to free cells [40].

E. Continuous fermentation with different dilution rate

Continuous system of immobilized cells is a promising technique that may improve solvent productivity with the potential of on-line separation technology [11]. However, previous studies of continuous and fed-batch fermentation found that there was an intrinsic hindrance to high butanol productivity [41]. Low substrate concentrations shifted the system into an acidogenic phase rather than solvents yield [42]. The dilution rate of continuous operation was a possible influencing factor. Alteration of the dilution rate would affect both utilization of glucose and growth of cells in immobilized system [43], result in sharply changes of ABE productivity and concentrations [1, 14]. Therefore, an opposite dilution rate plays an important role in ABE fermentation.

The result of continuous fermentation with different dilution rate is shown in Fig.6. In order to protect strains from butanol, the end-product inhibition effect, the continuous fermentation system was carried out with the precondition of butanol concentration in the fermentation broth below 10 g/L [5]. Initially, the medium was fed into the immobilized system at the dilution rate of 0.08 /h with 60 g/L glucose contained fresh medium. In this case, a maximized total ABE concentration in the fermentation broth was maintained at ~16.5 g/L (~9 g/L of butanol, ~6 g/L of acetone and ~1.5 g/L of ethanol). The productivity of ABE at the dilution rate of 0.08 /h was ~1.32 g/L·h. Gradually, with the dilution rate got higher, the residue sugar remained in the fermentation broth increased. At the same time, concentrations of ABE solvents decreased, which was similar with the tendency of previous studies of Survase et al and Zhang et al in using lignocellulosic immobilized carriers [14, 46]. When the dilution rate achieved 0.32 /h in present work, there still are ~40 g/L of glucose remaining unfermented. At the same time, the concentration of ABE solvents maintained at above ~7 g/L(~4 g/L of butanol, ~2 g/L of acetone and ~1 g/L of ethanol) while the highest productivity of ABE was obtained (~2.24 g/L·h) based on current test.

F. Comparison to other studies

Immobilized cells technology operated in the ABE fermentation process has been widely studied as an effective method to achieve

Table 1. Comparison of batch and continuous reactor performance with the different cell immobilization technique

Scenario	Supprot material	Strain	ABE production (g/L)	ABE yield (g/g)	ABE productivity (g/L•h)	Operation days	References
Batch	Alkali-treated steam-exploded corn stover	*C. acetobutylicum* ATCC824	16.95	0.32	0.24	-	25
	Brick	*C. acetobutylicum* BCRC 10639	~18	~0.31	0.17	-	49
	Sweet sorghum bagasse	*C. acetobutylicum* ABE 1201	24.4	0.44	0.42	-	This work
Continuous	Fibrous bed	*C. beijerinckii* ATCC 55025	8.5	0.53	7.6	22	44
	Wood pulp fibers	*C. beijerinckii* DSM 6423	5.22 (butanol and isopropanol)	0.30	5.22	27	46
	Brick	*C. beijerinckii* BA101	7.9	0.38	15.80	25	45
	Brick	*C. acetobutylicum* BCRC 10639	17.25	0.52 (butanol)	0.46	13	43
	Corn stalk	*C. beijerinckii* ATCC 55025	5.1	0.32	5.06	20	14
	Polyvinyl alcohol	*C. beijerinckii* NCIMB 8052	22.1	0.44 (butanol)	0.40	6.5	47
	Wood pulp	*C. acetobutylicum* DSM 792	12	0.27	4.86	-	48
	Sweet sorghum bagasse	*C. acetobutylicum* ABE 1201	16.5	0.43	1.32	41	This work

high butanol productivity and system stability, which has prospect in online butanol recovery and industrialization. Table 1 summarizes and compares recent studies on immobilized ABE fermentation in using different types of the strains. In general, cell immobilization can significantly increase the reactor productivity due to the increased cell density and the eliminate reactor downtime [5]. As superiority the immobilized technology has studies in-depth understanding of different carrier properties of microbial cells was carried out. Lignocellulosic biomass is considered to be the most cost effective and sustainable feedstock. They are more commonly being used as basic immobilization materials [50, 51]. In present study, sweet sorghum bagasse, the lignocellulosic residue of ethanol fermentation process, was salvaged without any chemical modification under environmental and economical consideration [17]. Besides, compared to other immobilized methods with average ABE yield of 0.3-0.4 g/g, sweet sorghum bagasse carrier showed better potential in sugar efficient conversion (0.43-0.44 g/g) in both batch and continuous scenario.

In addition, conventional ABE fermentation has the drawback of low butanol productivity, yield and titer, which led to biobutanol falling behind the petrochemical butanol in economic competitiveness [2, 52]. Integrating products recovery process with fermentation is a method to solve the problem [53]. In facing the need of in situ separation processes, immobilized fermentation process are calls for higher ABE production and ABE productivity simultaneously, in order to maximize the efficacy of process and minimize the cost of purification. Thus, in the present study, continuous fermentation process had balanced advantages in both ABE production (16.5 g/L) and productivity (1.32 g/L·h) at the dilution rate of 0.08 /h. As compared, other processes, however, held the unilateral advantages, which resulted in high product recovery cost (with low ABE concentrations) or low productive efficiency (with low ABE productivity).

To our best knowledge, immobilized process in using sweet sorghum bagaase carrier had the longest operation time (970 h) in continuous ABE fermentation scenario, which showed good stability and techno-economical feasibility of the process and eventually to follow a research strategy on an industrial scale. As compared, solvent production began to reduce only after a short time (140 h) in free cells continuous fermentation because of the poor surrounding conditions and poor pH tolerance of strains [54]. Thus, it was clear that ABE fermentation using sweet sorghum bagasse as immobilization carrier offered an efficient and stable way for the production of biobutanol.

IV. Conclusions

This study served a novel immobilization method of Clostridium to sorghum bagasse for butanol production. As a result, the

immobilized system enhanced ABE production and high ABE productivity. It was worth noting that the immobilized method would achieve a considerably high ABE yield of 0.43-0.44 g/g glucose. 10 batch of repeated fed-batch fermentation was performed continuously, with high stability of the system. In continuous scenario, a total of 970 h long terms of fermentation with different dilution rates showed balanced advantages in both ABE production and productivity when using sweet sorghum bagasse as immobilized carrier. The immobilized fermentation process maintained a stable productivity and high butanol yield for extending period, making the process attractive to industrial production of biobased butanol.

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Notes and references

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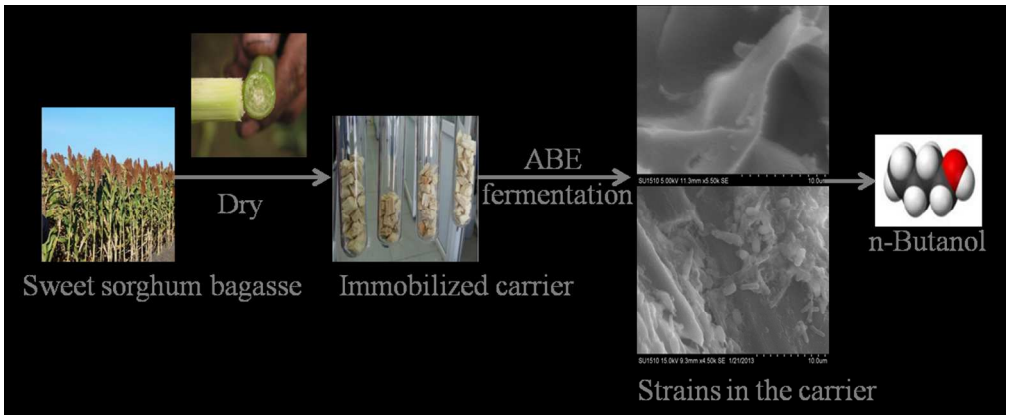

Sweet sorghum bagasse as an immobilized carrier for ABE fermentation by using Clostridium acetobutylicum ABE 1201
217x88mm (150 x 150 DPI)